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PHYSIOLOGICAL AND BIOCHEMICAL STUDIES
OF A MUTANT FROM GATEWAY BARLEY

by

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A DISSERTATION

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Physiological and Biochemical Studies of a Mutant from Gateway Barley" submitted by Raymond A. Miller in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

The effect of light quality, light intensity and temperature on the action of a virescens barley gene (yv_2) was investigated. All factors had a significantly different effect on chlorophyll accumulation in the mutant than in the normal barley line. The mutant was less susceptible to chlorophyll destruction by high light intensity, especially when grown at 29.0° . Low temperature had a highly detrimental effect on chlorophyll accumulation in the mutant, and large Q_{10} values were exhibited in the region of 14.0 to 19.5° .

Reversal of the mutant effect was attempted by the application of various chemicals. Although this was not attained, differential action on the two barley lines resulted from several treatments. Of the effective chemicals, the kinins appeared to offer some explanation of gene action.

The metabolism of amino acids and protein in the two lines grown in the dark was traced over a 9 day period. Little difference existed between the lines except for a slight reduction of protein synthesis in the mutant leaves. When grown under light, large differences were found in the alcohol soluble amino acids from the two lines, especially in asparagine. The lack of utilization of amide by the mutant resulted in an increased difference in the leaf protein synthesized by the two lines.

The total RNA content of mutant leaves was about 75% of that found in the normal, whereas the quantity of rRNA was 85 to 90%.

The data suggested either a difference in the state of rRNA, which affected the extraction by phenol, or considerably less than normal amounts of mRNA and sRNA in the mutant. The two barley lines showed no differences in their base ratios.

The results of the experiments are most readily explained by proposing that the mutation resulted in a change in the structure of mRNA responsible for synthesis of chlorophyll holochrome protein, rather than directly affecting chlorophyll molecule production.

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INTRODUCTION

The use of mutations in the study of metabolic processes in living organisms has become increasingly common in the last two decades. Whole new concepts, such as the operon theory concerning gene action, have been based on differential responses exhibited by organisms differing in one or a few genes. The use of such organisms has proved to be of particular value in the study of intermediary metabolism of microorganisms. In higher plants and animals the contribution has been less spectacular due to the involvement of more complex systems of metabolism, and to the difficulty in the propagation of the large populations necessary for accurate genetic and biochemical determinations.

Nevertheless, examples may be cited where important biochemical information has been gained in the study of mutants of higher organisms. In man, for instance, the study of hemoglobin from individuals affected with sickle-cell anaemia has led to a greater understanding of protein structure and function. Also studies of the abnormalities alkaptonuria and phenylketonuria have contributed to the knowledge of cyclic amino acid metabolism, and galactosemia and inherited diabetes provided a basis for the investigation of sugar metabolism in man.

One of the most extensively studied biochemical-genetical systems in plants is that concerning the production of pigments. The water soluble pigments have been investigated particularly extensively, and a series of mutants used to chart the pathway of biosynthesis of these compounds. A few attempts have been made to use such a series

of mutants to investigate chlorophyll production in plants. Evidence gained has indicated that chlorophyll a and b have a common precursor, and in fact the genetic evidence suggests that chlorophyll a acts as a precursor for chlorophyll b. Much, however, remains to be learned about this system.

The major obstacle preventing the wider use of mutants in the study of metabolic sequences is the lack of detailed physiological and biochemical knowledge of the mutants which are available. The present study was undertaken for the purpose of obtaining such information on a single gene, chlorotic mutant of barley. This mutant is of the virescens type, which begins growth in a highly etiolated condition but gains in greenness as growth progresses. Since it was noted during the genetic studies that the mutant responded strongly to environmental conditions, it was selected for physiological and biochemical studies because of the possibility of controlling the gene expression at will by altering the conditions of growth. A mutant of this type would be of particular value in the study of processes leading to the production of mature chloroplasts containing a stable chlorophyll holochrome. Realization of this objective was not expected of the present work, but rather the plan was to gain further information basic to the gene action which could be utilized in future experiments.

LITERATURE REVIEW

I. Pigment Studies

1. Effect of Light Quality

The first action spectrum for chlorophyll accumulation was obtained by Schmidt (Smith and Young, 1956) using etiolated corn seedlings. His results indicated 3 peaks of effectiveness; at 640, 567 and 450 m μ . Since the early work of Schmidt there have been a number of reports concerning the effect of wavelength on chlorophyll accumulation and on the conversion of protochlorophyll to chlorophyll. Differences in the action spectra for these two phenomena (Smith and Young, 1956) indicate that they should be considered independently. Since the experiments in the present study were of long duration, only chlorophyll accumulation need be considered.

Several reports indicate that the red region is most effective in promoting chlorophyll accumulation (Polishchuk and Mindel, 1962; Ruben and Chernavina, 1955; Smith and Young, 1956), while others report the blue to be most effective (Appleman and Pyfrom, 1955; Frank, 1946). The results shown by Frank have been criticized on the basis that they are supposed to represent an action spectrum for protochlorophyll conversion to chlorophyll (Smith and Young, 1956). Since the period of exposure was 5 hours, this represents a sufficient length of time so that the results may be interpreted as partially representing chlorophyll accumulation rather than just protochlorophyll conversion, even though the light intensities were low.

A similar conflict exists in the literature concerning the relative effectiveness of blue and red light on the accumulation of carotenoids (Rudolph, 1933; Sargent, 1940; Brandt, 1958). Considerable difficulty may arise in this measurement due to the use of plants having different amounts of chlorophyll. Since it has been clearly shown that light stimulates carotenoid production, and since absorption of light by the leaf would be dominated by chlorophyll, a variation in chlorophyll content under light of low intensity would likely result in a strong influence on carotenoid production (Smith and Young, 1956). It must also be remembered that chlorophyll accumulation is an autocatalytic process (Smith and French, 1963).

In addition to the above conflicts, Kakhnovitch (1961) points out that if the incident energy is greater than $20,000 \text{ ergs/cm}^2$ per second, wavelength has no differential effect on pigment accumulation.

2. Effect of Light Intensity

Although the light intensity necessary to bring about chlorophyll formation is very low, the data presented by Lubimenko (1928) indicated that a threshold intensity does exist, below which chlorophyll will not be formed.

The initial production of chlorophyll in etiolated plants is directly proportional to light intensity at relatively low incident energy, but this relationship is not extended over long-term studies or to high intensity illumination (Scharfnagel, 1931; Smith and Young, 1956). In long-term experiments the determination of the most effective intensity is complicated by differences in the accumulation

of photosynthate (Sorokin, 1960) or by leaf expansion caused by different intensities. Thus Sargent (1940) found that Chlorella grown at high light intensity contained one-half the chlorophyll per cell of those grown at one-seventh the intensity, but that the culture as a whole would produce 2.5 times as much chlorophyll at the higher than at the lower intensity. Myers (1946) found the optimum intensity for chlorophyll production by a culture of Chlorella to be 55 ft-c.

Shirley (1929) using a number of species grown under 74% to 1% of the intensity of sunlight, noted an increase in chlorophyll content per unit weight of leaf through the intensity range 74% to 8%. One per cent appeared to be below optimum intensity.

Using Marquis wheat, Friend (1960) observed an increase in chlorophyll content in the first 3 leaves through the intensity range 200 to 2500 ft-c provided the temperature was 20 to 30°. This pattern was obtained whether the chlorophyll content was calculated per leaf or as a percentage of the fresh leaf weight. At temperatures lower than 20° the optimum intensity was less than 2500 ft-c. There was, however, a consistent increase in chlorophyll content at all temperatures when the intensities were increased from 200 to 1000 ft-c. The optimum conditions for chlorophyll accumulation on the basis of leaf weight were 2500 ft-c and 30° C, although little difference existed in the range 20 to 30° at 2500 ft-c.

It is apparent from the comparison of Friend's and Meyer's data that the optimum intensity for chlorophyll accumulation is highly species dependent, probably reflecting the adaptation of species for particular environments.

Although most plants produce carotenoids in the dark, light stimulates this production (Wolf, 1963; Kay and Phinney, 1956). Frank and Kenney (1954), however, reported a reduction of total carotenoid in corn leaves upon exposure to light. In any case the main effect of light appears to be on the relative composition of the carotenoid content. Wolf (1963) found that in the dark carotene comprised only 7.6% of the total carotenoids in wheat seedlings, whereas in the light this proportion rose to 33%. A very rapid production of carotene in corn seedlings when exposed to light was also indicated in the experiments of Kay and Phinney (1956). Similar results were obtained by Strain (1938) in barley seedlings. Bandurski (1949) concluded that the rapid synthesis of carotenes was dependent upon the availability of photosynthate. Goodwin and Phagpolngarm (1960) however, state that there is no specific synthesis of β -carotene in bean seedlings when exposed to light, but instead there is a synthesis of all carotenoids associated with the development of chloroplasts.

The effect of light intensity on total carotenoid production is similar to its effect on chlorophyll production (Brandt, 1958). At low light intensity the production of carotenoid is proportional to the intensity, but there also appears to be an optimum intensity since Moster and Quackenbush (1952) found a marked decrease in the carotenoid content of corn seedlings grown at 2500 ft-c as compared to those grown at 500 ft-c. The earlier results of Seybold and Egle (1938; ref to by Goodwin, 1962) also indicated that lower carotenoid levels in leaves were brought about by high light intensity.

3. Effect of Temperature

Sacks in 1865 (ref. to by Smith and Young, 1956) was the first to report that a threshold temperature exists for chlorophyll formation, and that the rate of greening increases with temperature. Virgin (1955) obtained similar results for protochlorophyll formation in etiolated barley seedlings over the temperature range 0 to 30⁰. Lubimenko and Hubinet (1932) found the optimum temperature for chlorophyll accumulation per gram fresh leaf weight of etiolated wheat seedlings to be about 26⁰ for long-term exposures. Friend's data (1960), however, indicated an optimum of at least 30⁰. Although much of the earlier data was suggestive, the first statistical proof of an interaction of light and temperature on chlorophyll accumulation was also presented by Friend.

Moster and Quackenbush (1952) found a marked increase in the carotenoid content per gram fresh weight of corn seedlings when the temperature was lowered from 20 to 5⁰, regardless of whether the material was grown in light or darkness. There was essentially no difference in the level of carotenoids at 20 and 35⁰. The temperature inhibition studies on carotene synthesis in tomatoes carried out by Tomes (1963) indicated two possible pathways of carotene synthesis. These two pathways responded quite differently to temperature inhibition.

4. The Effect of Various Chemical Treatments

In higher plants few cases have been reported where the reversal of the affect of a mutant gene has been brought about by

an external application of a chemical. This was, however, accomplished by von Wettstein (1961) with two chlorotic mutants of barley; an albino and a xantha. An application of aspartic acid to the albino and leucine to the xantha enabled the mutant seedlings to produce chlorophyll. A similar reversal was effected by gibberellic acid on certain single-gene dwarf mutants of corn in the studies by Phinney (1956).

Numerous studies have connected chlorosis in plants with various mineral deficiencies.

Although a great deal of study has been directed towards the elucidation of the effects of iron deficiency in plants, little is known about the biochemistry of this phenomenon. The study of iron deficiency is complicated by the requirement of this element in a particular form within the plant. Thus a deficiency has been noted in cases where the chlorotic plants actually contained more iron than normal green plants (Wallace, 1962). An application of iron resulted in a correction of the chlorotic condition. Also iron deficiency has been noted to result in some loss of activity of iron-containing enzymes, especially catalase and peroxidase, in spite of the presence of much more iron than necessary for the synthesis of enzymes (Wallace, 1962).

Marsh et al. (1963) proposed that a deficiency of iron hindered the formation of δ -amino levulinic acid as well as the conversion of protoporphyrin to heme — steps involved in chlorophyll synthesis (Smith and French, 1963). Marsh et al. also showed that

at least in cowpeas there is no effect of iron deficiency on the steps leading from δ -amino levulinic acid to protoporphyrin. Carell and Price (1965) also found that the conversion of coproporphyrinogen to protoporphyrin in Euglena was not affected except at extreme iron deficiency.

The susceptibility of plants to iron chlorosis can be affected by the genetic constitution. Different varieties of beans (Brown et al., 1961) and two lines of corn differing by one gene (Bell et al., 1961) responded differently to ferric but not to ferrous iron. Brown demonstrated that the roots of the bean variety most susceptible to iron chlorosis had a much lower reductive capacity than did the less susceptible variety. This agrees well with Kliman's suggestion (1937) that iron must be in the reduced state before it can be taken up by the roots.

Since magnesium is an integral part of the chlorophyll molecule a deficiency of this mineral would obviously lead to chlorosis. Symptoms of magnesium deficiency are first noted in the older leaves since this element is transferred to younger developing tissues (Curtis and Clark, 1950).

A deficiency of each of the micronutrients - boron, cobalt and copper - has been shown to cause chlorosis (Heinonen, 1961; Meyer and Anderson, 1939). Little is known of the role they play in chlorophyll accumulation but it is most probable that they act in an indirect manner as adjuvants to enzymes.

Gibberellic acid, kinetin and N⁶-benzyladenine have all been reported to act as chlorophyll stabilizers in detached leaves or senescing tissue (Brian et al., 1959; Richmond and Lang, 1957; Zink, 1961; Wittwer et al., 1962, 1963; Alvim, 1962). The exact mechanism of action of these chemicals is unknown, although many studies have linked the kinetin and N⁶-benzyladenine to increased contents of protein and ribonucleic acid in treated tissues (Tsujita, 1964).

The first person to measure the increase in chlorophyll content of plants due to a treatment with (2-chloroethyl) trimethylammonium chloride (CCC) was Humphries in 1963. Norris (1964) also noted a substantial increase in the chlorophyll content of corn and brome grass 5 to 8 weeks after treatment with CCC. The effect was particularly marked if the chlorophyll content is considered on the basis of leaf area since this chemical also causes a substantial reduction in leaf length. In spite of this reduction, however, the chlorophyll content per leaf was increased in corn seedlings when grown in a solution of 1000 to 2500 ppm of CCC.

Gramine has been shown to occur in a chlorophyll deficient barley mutant (Wightman et al., 1961). However, it was not shown whether the gramine was produced as a direct result of the mutation and then acted as a toxic factor bringing about the chlorotic condition, or whether the chlorosis was caused by a fault in some other metabolic process and the gramine produced as a side effect.

Benzimidazole has repeatedly been shown to retard chlorophyll degradation in detached leaves (Wang, 1961). King et al. (1963) found that benzimidazole maintained the protein level of detached leaves by enhancing protein synthesis. It is possible, therefore, that this

chemical acts on chlorophyll stabilization through its influence on the protein level in the leaf, since it is well known that the stability of chlorophyll is dependent upon the formation of a complex with protein.

A number of reports (to be discussed in a later section) have linked abnormally high amounts of asparagine and glutamine with chlorosis in leaf tissue. It is generally considered that these amides are formed as a method of detoxification of ammonia which is released during protein catabolism (Boulter and Barber, 1963). Demetriades (1956) has shown that large amounts of amide accumulate in the embryos of normal etiolated seedlings during germination. He suggested that this accumulation in etiolated seedlings, as well as that which was found in chlorotic leaves, is the result of the inability to synthesize protein. This has been substantiated by Perur et al. (1961) who found an 82% reduction in the chloroplastic protein of corn leaves suffering from iron deficiency.

II. Amino Acid Composition of Seeds and Seedlings

1. Free Amino Acids and Amides

As reported by Maclachlan and Zalick (1963) the leaves of the barley mutant (yv_2) seedlings have a very high free amino acid content in comparison to those of normal Gateway barley. A number of other conditions have been noted to bring about the same phenomenon in other plants. Among these are deficiencies in copper, zinc, manganese, iron, sulfur, phosphorus, potassium (Yemm and Folkes, 1958), etiolation

(McKee, 1962), tumor growth and viral infection (Hagen and Gunkel, 1958). Demetriades (1956) concluded that the high free amide content in seedlings suffering from iron deficiency or from etiolation was due to the inability of the plants to utilize the amide which is produced during the normal process of germination.

The free amino acid content of the embryo does not reflect the composition of the leaf protein (Thompson and Steward, 1952). In addition the peptide fraction in the embryo bears little resemblance to either the free amino acid or protein composition, but it does resemble the peptide in the endosperm which in turn reflects the composition of the storage protein (Yemm and Folkes, 1958). Thus it is probable that partial-hydrolysis products of stored protein can be transported to the developing embryo as suggested by Sampaio and Folkes (Yemm and Folkes, 1958) but are not incorporated directly into new protein. Ledoux (1962) has obtained evidence that intact proteins may also be transported from the seed to various developing organs. and Beevers
The work of Oaks[^] (1964) has clearly shown that a supply of organic nitrogen in the form of amino acids is required for normal synthesis of protein in maize embryos. Similar results have been obtained with oat (Harris, 1955) and barley seedlings (Folkes, 1959). The latter two species grow very little when the embryo is separated from the endosperm at an early stage of germination.

Work by Yemm (1949) provided the first direct evidence that asparagine synthesis in barley leaves is associated with the breakdown of reserve protein in the seed. Earlier work by Dunn (1948) using soybeans and lupine seedlings also indicated a large increase in

aspartic residues at the expense of other amino acids, especially glutamic, from the reserve seed protein. Panalaks et al. (1963) concluded that the asparagine in the cotyledons of soybean seedlings came either directly from hydrolysis of reserve protein or from the amidation of asparatic acid residues released by hydrolysis during germination. The large amount of asparagine found in the plant axis, however, was thought to be synthesized de novo at the expense of glutamic acid transported from the reserve protein in the cotyledons.

Light has been shown to have a high stimulatory effect on nitrate reduction (Kessler, 1964) and Naylor and Tolbert (1956) report that glutamic acid in barley leaves undergoes transformations much more rapidly in light than in darkness. Similar results were obtained by Kretovich (1959) using homogenates of green and etiolated pea seedlings.

A high accumulation of free amide nitrogen has been associated with an initial low level of sugar in detached barley leaves placed in the dark (Yemm, 1949). The majority of this amide was asparagine. A higher content of sugar favoured the accumulation of glutamine at the expense of asparagine, and the total amide level was much less than in the leaves having an initial low sugar content. Thus there appears to be some connection between the level of available carbohydrate and quality as well as quantity of amide accumulated in detached leaves.

Although etiolation in barley and many other plants promotes a high accumulation of asparagine and relatively much less glutamine,

the situation in cotton seedlings as reported by Karavaeva (1962) appears to be the opposite. In the dark glutamine accumulated at the expense of asparagine, while in the light the process was reversed.

Folkes and Yemm (1958), using actively growing seedlings exposed to light, point out that very little free glutamine or glutamic acid is present in the embryo, and that a far greater amount is associated with the peptide fraction. The same was true for proline, methionine, histidine and arginine, so that in some cases these amino acids were present only in trace amounts or were completely undetected in the free state. Bollard (1956), however, found that glutamine and asparagine were the most plentiful nitrogenous compounds in the xylem sap of many plant species. Thompson and Steward (1952) reported that 78% of the alcohol soluble nitrogen from potato tubers was in the form of amides.

Although glutamine enters transamination reactions in mammalian cells (Mardashev, 1951) using the amide nitrogen to produce asparagine, this reaction could not be demonstrated in lupine or wheat germ (Webster[^] and Varner 1955). However, the rapid turnover of glutamine in plant cells (Meister, 1955) indicates that the nitrogen of glutamine is mobile and therefore available for synthesis of other amino acids. The same is true for asparagine although it enters reactions much more slowly than does glutamine (Meister, 1955). Vickery (1963), using excised tobacco leaves in darkness, showed that 26% of N¹⁵-asparagine taken up was metabolized in 48 hours, even though there was a net synthesis of asparagine during the test period. Kretovitch (1959) suggested that the slower rate of asparagine utilization for

protein synthesis as compared to that of glutamine, may be due to the in vivo formation of cyclic asparagine. Kretovitch's experiments indicated that the ring structure forms only under aerobic conditions, thus leading to the suggestion that the rate of asparagine catabolism is dependent upon the oxidation-reduction potential of the cell contents. This hypothesis is compatible with the observations that asparagine accumulates in the dark and disappears in the light. It is possible, however, that in plants amide nitrogen does not enter directly into transamination reactions.

The particular importance of glutamic acid in the synthesis of protein was stressed by Folkes (1958) who suggested that as many as 16 of the other common amino acids may be synthesized via transamination by glutamic acid. Other results using N^{15} (Wilson and Burris, 1953; Allison and Burris, 1957) indicated that glutamic acid may be the only primary product of inorganic nitrogen incorporation into the plant. The labeling indicated that nitrogen is transferred rapidly to form secondary products such as glutamine, aspartic acid, asparagine and alanine.

Although it is generally assumed that much of the amino acid synthesis takes place by amination of the corresponding α -keto acids, Nelson and Krotkov (1956) using C^{14} tracer studies, concluded that it was unlikely that glutamic and aspartic acids were synthesized by such a mechanism in wheat leaves. These authors also concluded that glutamine and asparagine were synthesized from the corresponding acids, but Lerman and Mardeshev (1960) using N^{15} reported that α -keto succinate was a more likely precursor of asparagine than was aspartic acid.

On the basis of a difference in the pattern of labeling of the free amino acid pool and newly formed protein in barley leaves, Folkes (1959) suggested the existence of two free amino acid pools. He proposed that one of these may be a storage pool, presumably in the vacuole, and the other a metabolically active pool. Using $C^{14}O_2$ Hellebust and Bidwell (1963) found it possible to calculate the proportions of some protein amino acids which came either from the free pool or by a direct route from photosynthate to protein. The results indicated that the largest part of the protein bound glutamic, aspartic and alanine came from the storage pool, whereas more than half of the serine and glycine came via a direct pathway from photosynthate. It is possible that the "direct route" may be through a free amino acid pool which is quite small and isolated from the free pool by being located in the chloroplasts. Further evidence for the existence of more than one free amino acid pool was given by Oaks (1965a). She found good evidence of two free leucine pools in maize root tips. Cases of similar phenomena have been reported in lower organisms. An expandable as well as a non-expandable metabolically active tryptophan pool was shown to be present in Neurospora crassa by Matchett and De Moss (1964). Tryptophan synthesized from indole entered only the metabolically active pool, which then acted as a protein precursor pool. Similarly the studies by Sercarz and Gorgini (1964) on Escherichia coli indicated two pools of free arginine having different functions.

2. Amino Acid Composition of Barley Seed Protein

Often the first noticeable change during germination is the shift in the ratio of protein to soluble nitrogen. The hydrolysis of storage protein is probably selective since the globulins of chickpeas are readily broken down while the albumins remain fairly constant but altered in nature (Koller^{et al.} 1962). Although the rate of utilization of the various proteins in barley endosperm differs, essentially all the reserve protein is utilized by the tenth day of germination (Folkes and Yemm, 1958). In his monograph Djurtoft (1961) presents a comprehensive review of the nature of proteins in barley seed.

In table 1 is given the content of some amino acids found in barley seed by several groups of workers. The original data have been recalculated and expressed as a molar percentage of leucine. Different varieties, growth conditions, and analysis procedures apparently result in considerable variability in the values given. In general the greatest discrepancies are between Baumgarten's data and that of the other workers. It is possible that the differences are due to a high value for leucine (and isoleucine) in Baumgarten's determinations.

Folkes and Yemm (1958) traced the amino acid composition of various protein fractions of germinating barley from 0 to 10 days. No external nitrogen was supplied to the seedlings. It was possible to draw up balance sheets for some of the amino acids and part of their data is given in tables 1 and 2. The data shown are for a variety of barley having a high nitrogen content (2.6%) and includes all the

Table 1

Amino Acid Composition of Barley Seed
Molar Content Expressed as a % of Leucine

	Renner <u>et al.</u> (1953)	Horn <u>et al.</u> (1955)	Baumgarten <u>et al.</u> (1946)	Folkes and Yemm (1958)	Folkes and Yemm (1958). Content of total seedlings after 10 days development. Expressed as % of seed content.
Amide				320	39
Lysine	43	48	36	39	186
Histidine	24	26	25	22	110
Arginine	58	53	38	54	118
Aspartic				81	154
Threonine	52	57	44	51	129
Serine			62	71	83
Glutamic			285	326	45
Proline				206	38
Glycine				88	131
Alanine				77	170
Valine	76	87	78	84	100
Methionine	15	18	18	19	107
Leucine	100	100	100	100	99
Isoleucine	58	60	70	70	105
Tyrosine			14	35	106
Phenylalanine	65	61	56	61	83
Tryptophan			13	18	166

Table 2

Balance Sheet for Proline and Valine at Different Stages of Seedling Development. From Yemm and Folkes, 1958.

Day	Whole Plant		Endosperm		Embryo	
	Proline	Valine*	Proline	Valine*	Proline	Valine*
2	96	95	96	93	114	114
4	63	80	52	50	368	362
6	43	85	20	20	672	688
8	39	100	8	10	911	938
10	35	93	2	4	922	914

* Valine measurements do not include "free" acid. All the data has been recalculated and expressed as a percentage of the content on day 0.

residues from protein in the seed or seedling. In addition the data for amide, aspartic, glutamic, proline, lysine, histidine and arginine includes the free residues and those from peptides. The major part of the nitrogen was stored in the form of amide nitrogen, glutamic acid, proline and arginine. During germination in the light there was a rapid consumption of stored proline, glutamic acid and amide nitrogen, and a correspondingly rapid increase in the acids aspartic, glycine, lysine and arginine. Over a ten day period small increases were noted in tryptophan, threonine, isoleucine, histidine and small decreases in serine and phenylalanine. At the end of the 10 day test period less than 5% of the original nitrogen remained in the endosperm. In the fractions measured there was about 13% reduction

in the nitrogen content. This loss of protein nitrogen was probably present in the form of free amino acids and peptides not included in the data, or it may have been utilized for the synthesis of nucleic acids.

Yemm and Folkes (1958) state that more than 70% of the amino acids necessary for the developing embryo are contained in the endosperm. Additional amounts of aspartic, glycine, lysine and arginine are needed and are synthesized at the expense of excess glutamic acid and proline as well as amide. In addition the embryo is required to supply nitrogen for the synthesis of nucleic acids and chlorophyll, although Ledoux (1962) found that 90% of the new RNA in the embryo could be accounted for by translocation from the seed.

3. Amino Acid Composition of Leaf Protein

and Beevers
Oaks_A(1964) found that isolated corn embryos grew well without an external supply of organic nitrogen but that the protein contents were greatly reduced. The production of normal quantities of protein appeared to be dependent upon a supply of amino acids from the endosperm. An addition to the growth medium of a leachate from endosperm completely restored protein synthesis in the isolated embryos. A synthetic mixture of amino acids having the same composition as the hydrolysate of the leachate was also fully effective. The critical residues were found to be contained in the basic and neutral amino acid groups, and normal quantities of protein were produced by the embryos when no pre-formed glutamic or aspartic acid was supplied. After 108 hours of germination the level of the acidic acids and

their amides in isolated embryos was equal to that found in embryos of intact seedlings. Thus it appears that synthesis of the acidic amino acids is carried out efficiently even in very young embryos. Oaks suggested that the lack of protein synthesis in the isolated embryos was due to a lag in the ability to produce complicated amino acids. After a suitable inductive period this ability developed and protein synthesis proceeded normally.

The situation may be similar in barley since Jay (Folkes, 1959) using isolated embryos under aseptic conditions, found that nearly normal development could be attained if the nutrient culture contained a "well balanced" amino acid mixture in addition to sucrose and inorganic salts. Somewhat the same results were obtained by Harris (1955) using isolated oat embryos.

Protein synthesis in chloroplasts was demonstrated by Degen-Grenson (1954). Yemm and Folkes (1953) reported that about 39% of the total barley leaf protein is in the chloroplasts. Amino acid analysis of chloroplastic and cytoplasmic proteins from spinach leaves was carried out by Chibnall (1939), Noack and Timm (1942), Timm (1942), and Stoll and Wiedemann (1952). Yemm and Folkes (1953) compared the amino acid composition of the cytoplasm plus chloroplasts with that of isolated cytoplasm. Although there was some disagreement in the absolute values obtained by the different workers, these analyses indicated that the only major difference in the composition of these two groups of proteins was in the content of lysine, the protein from chloroplasts having a lower proportion of lysine than does protein from cytoplasm.

Noack and Timm also reported that chloroplastic protein from spinach had a somewhat higher proportion of glutamic acid and histidine; however, Yemm and Folkes did not find this to be so in barley leaf. In addition, Stoll and Wiedemann reported a much lower content of arginine in purified chloroplastin than the earlier studies did in the total chloroplastic protein.

The possibility that the synthesis of specific leaf proteins is affected by conditions which cause high free amino acid and amide concentrations is indicated by a number of investigations (Peruer, 1961; Lyttleton, 1962; Brawerman, 1962 a and b). Iron chlorosis of corn (Perur, 1961) caused an 82% reduction in the protein content of the chloroplastic fraction of the leaf, whereas the protein concentration in the cytoplasm was apparently not changed. Heber (1962 a, b) has shown that light stimulates a rapid incorporation of C^{14} into chloroplast proteins in spinach leaves. In addition the lamellar fraction is labeled some 10 times more heavily than are the soluble proteins from the chloroplasts. These observations have been substantiated by the studies of Benno (1964) and Osipova et al. (1964). Early investigations using C^{14} to study CO_2 fixation during photosynthesis indicated that the amino acids alanine and aspartic are among the first compounds to become labeled when leaves are exposed to $C^{14}O_2$ in the light (Benson et al., 1950). Andreeva et al. (1962) showed that both the free amino acid and protein composition of sunflower leaves was altered by active photosynthesis. Heber (1962b) clearly showed that synthesis of amino acids can take place in the chloroplasts during photosynthesis. Thus it appears that light not only causes the

production of amino acids and stimulates protein synthesis in leaves, but that under suitable conditions it stimulates a highly specific synthesis of proteins which are involved in structures within the chloroplasts. It has been suggested by Molotkovskii et al. (1963) that light induces the synthesis of a specific ribonucleic acid which then acts as a template for the synthesis of chloroplastic protein, thereby establishing the basis for subsequent chlorophyll accumulation.

III. Ribonucleic Acid (RNA) in Leaf Tissue

In the last decade a number of reviews have been written on the role of nucleic acids in living organisms. Most of the experimental work cited has been heavily orientated towards bacterial, viral and animal systems; however, the fundamental principles are thought to be similar in all organisms (Umbarger, 1963). The reader is referred to the excellent articles by Watson (1963), Nirenberg and Leder (1964), and Commoner (1964) for detailed information on the functional aspects of nucleic acids, and to those by Stebbins (1965) and Umbarger (1963) for a broader view of the role played by these compounds in the controlling of processes leading to differentiation of an organism.

Ribonucleic acid is composed of 4 nucleotides, or derivatives of these, linked together by a phosphate bridge between the 2 and 5 positions of the ribose moiety. The 4 nucleotides are: adenylic acid (Ap), guanylic acid (Gp), cytidylic acid (Cp) and uridylic acid (Up), the first two contain purine bases and the latter two pyrimidine bases.

There are at least 3 types of RNA, each having a different function in the cell. Soluble ribonucleic acid (s-RNA) is associated with the activation of amino acids prior to incorporation into protein. Messenger ribonucleic acid (m-RNA) is that type of RNA which mediates genetic information between DNA and enzymes. It is thought that DNA acts as a template for m-RNA synthesis, which then migrates to the sites of protein synthesis — the ribosomes. Here the m-RNA in turn acts as a template for protein production. The key to the template system resides in the base sequence of the nucleic acids, with the fundamental unit probably a triplet of bases.

The ribosomes themselves are composed of 40 to 60% RNA (r-RNA). The exact function of the r-RNA has not been determined in spite of this fraction constituting the largest portion of RNA in cells.

Unless specifically stated otherwise, future reference to RNA will include the total RNA of the cells.

Since the methods of analysis of nucleic acid composition in animal and bacterial tissues are not readily applicable to analysis of plant RNA due to the presence of interfering substances, much study has been directed towards methodology for nucleotide analysis of plants (DeDeken-Grenson and DeDeken, 1959; Smillie and Krotkov, 1960a; Nieman and Paulson, 1963; Ingle, 1963; Zscheile and Murray, 1963). Although some of the techniques employed indicated very good precision, the accuracy must remain in question until further comparisons are made.

The results obtained by several authors for the nucleotide composition of RNA hydrolysates from plant leaves are given in table 3. It may be seen that differences in RNA composition occur between species. The greatest variation appears to be the low values of Cp, and the correspondingly high values of Up, in Thomas' results for clover and rye as compared to the results given for the other species.

Table 3

Nucleotide Composition of RNA Isolated from Plant Leaf Tissue

Values are given as moles/100 moles nucleotides

Plant	Ap	Cp	Gp	Up	Pu/Py	Ref.
Clover	26.4	18.5	26.0	29.2	1.10	Thomas, 1956
Rye	24.2	20.6	29.0	26.0	1.14	Thomas, 1956
Tobacco	23.5	23.8	33.1	19.5	1.31	Reddi, 1957
Barley (Atlas)	22.1	22.4	33.3	22.2	1.24	Zscheile, 1963
<u>Euglena</u> (Green)	24.6	25.3	29.5	20.6	1.18	Brawerman, 1959
<u>Euglena</u> (Colorless)	22.7	26.6	29.9	20.7	1.11	Brawerman, 1959

The physiological condition of the organism may influence RNA composition as indicated by the values for green and colorless Euglena. Brawerman (1959) considered these results as indicative of specific RNA being synthesized in response to a specific stimulus, a suggestion which Molotkovskii and Moryakova (1963) have also invoked to explain the role of nucleic acid in their studies on induced chlorophyll synthesis.

Smillie and Krotkov (1960b) indicated that the RNA content of Euglena with fully developed chloroplasts is slightly higher than in those cells having only partially developed chloroplasts, whereas Brawerman et al. (1962b) indicated that colorless Euglena cells contain about 72% as much RNA as do green cells. Brawerman (1962a) concluded that chloroplastic ribosomes had a different base ratio than cytoplasmic ribosomes. Also Kessler and Engelberg (1962) found rather large seasonal changes in the RNA content of apple leaves. On the basis of fresh leaf weight maximum RNA content was reached in 3 day-old leaves. Over the next 7 days the RNA decreased gradually and nearly linearly.

On the basis of Zscheile's results (table 3) it may be calculated that the content of RNA in barley leaf is about 7.8 mg/g dry tissue. However, the age of the leaves used in this study was not given; it may be presumed that they were fully developed. Maclachlan and Zalik (1963) reported values of 3.1 and 2.5 mg free nucleotides/g fresh leaf of 12 day-old normal and mutant (yv_2) Gateway barley seedlings. These values must be held in question since they were determined on the basis of ribose measurements, a method which is generally considered quite unsatisfactory for plant tissue.

MATERIALS AND METHODS

I. Source of Experimental Material

The barley mutant used in this study has been described as being of the virescens type (Maclachlan and Zalik, 1963). That is, the seedlings at early stages are pale green or yellow but gain in greenness as they grow older. However, this mutant, like some of those described by Holm (1954), responds very differently to glass-house and field conditions in the Edmonton area. Under field conditions the mutant plants are yellow to ivory on emergence and remain so up to ripening. There is also extensive seedling lethality and those plants which do survive are very weak. Few tillers are produced and these are usually highly sterile (Walker et al., 1963). Thus although in the glasshouse the mutant acts like a virescens type, under field conditions it is more like xantha, consequently seed increase was always carried out in the glasshouse.

The mutation may be termed a spontaneous one since it arose in the water control of a chemical mutagenic study. The mutation involves a single gene, designated yv_2 , and the gene location was given as 23 cross-over units to the left of the brachytic locus on chromosome one (Walker et al., 1963).

The parent variety, Gateway, was used as a control material throughout the experiments. In all cases only seed produced by the two barley lines grown simultaneously was used.

II. Apparatus for Growth

1. Effect of Light Quality on Pigment Production

The cabinets and light filters which constituted the different treatments were described in detail earlier (Fletcher, 1964; Zalik and Miller, 1960). The wavelengths at 50% transmission, and the color designations given by Fletcher are included in table 4. The color designations are arbitrary and were given for convenience of reference rather than for accuracy of description. Thus the filter designated as green might be better described as blue-green, and the yellow as yellow-green, and so on. Ashton (1965) has reported slightly longer wavelengths at 50% transmission in a similarly constructed system.

The intensity of illumination in each cabinet was 2900 ergs/cm² per second as measured by a pyroheliometer (Fletcher, 1964). This intensity was obtained by adjusting the shelf height within the cabinets. The light source was 8 incandescent lamps for each cabinet, varying from 10 watts for the white control to 1000 watts per lamp for the blue treatment. A refrigerated, rapid air flow through the cabinets maintained the temperature at 20⁰. Heat from the lamps was dissipated by a separate air flow which was drawn over the lamps and then exhausted from the room, and also by a cold water flow over the filters.

2. The Effect of Light Intensity and Temperature on Pigment Production

Two of the cabinets used in the light quality experiments were divided vertically into 2 compartments. Four intensities of 320, 1020, 1800 and 2600 ft-c were obtained by varying the distance between the shelves and the light source. Baffles were arranged in the cabinets so that the plants were essentially illuminated only from the top, or by reflected light from the baffles. Light was provided by 300 watt incandescent lamps and heat energy was filtered out by approximately 13 cm of water. The light intensities were measured with a Weston light meter about 3 cm above the soil surface. A refrigerated air supply provided an ambient temperature of 14° in one of the chambers. Mean temperatures of 19.5, 24.5, and 29.0 were obtained in the other three chambers by adding an appropriate amount of hot air of a constant temperature to the refrigerated air flow. These temperatures, based on the temperature of water in stoppered erlenmeyers placed on each shelf, were read 3 times each day. Maximum variation was $\pm 1^{\circ}$.

3. The Effect of Various Chemicals on Pigment Production

This study was carried out in a commercial growth cabinet (Fleming-Pedlar) under carefully controlled environmental conditions. A light intensity of 1500 ft-c was provided by daylight type fluorescent lamps. The temperature of the circulated air was $19 \pm 1^{\circ}$, and the humidity was 50%.

4. Amino Acid Investigations

In the studies involving only etiolated material, the seedlings were grown in a germinating cabinet at constant temperature. Where different temperature and a light treatment were given, the cabinets already described in the study of the effect of light intensity and temperature on pigment production were utilized.

5. Ribonucleic Acid Measurements

Plant material for these experiments was germinated and grown in the Fleming-Pedlar growth chamber at 1500 ft-c, $19 \pm 1^{\circ}$ and 50% relative humidity.

III. Germination and Seedling Production

1. Effect of Light Quality on Pigment Production

Seeds of both lines were spread on trays of vermiculite, wetted with distilled water, covered with absorbent tissue, and placed in a germinating cabinet in the dark at 20° . After 24 hours they were selected for uniformity of development and transferred to 6 inch plastic pots containing California mix (1 peat:1 sand plus added K, N, P, Ca, Mg, and S). Initially each pot contained 15 seeds. After an additional 2 days at 20° in darkness the seedlings were again selected for uniformity and the pots then transferred into the various light treatments. The timing of this transfer was such that most of the coleoptiles had broken the soil surface. Continued selection was carried out during the experiment to eliminate as much variability as possible.

The seedlings were allowed to develop for 5 cycles of 8 hours light and 16 hours darkness at 20⁰. The seedlings were harvested at the end of the last dark period. Exposure to light during harvesting was such that protochlorophyll synthesized during the last dark period was converted to chlorophyll. Three to 10 primary leaves constituted a sample. Three samples were taken for each replication, and the studies were replicated after a time lapse. The leaves were cut into approximately 3 cm lengths, placed into glass vials and freeze-dried for 3 days at room temperature. The leaf tissue was then stored over P₂O₅ at -28⁰ for a minimum of 3 days before analysis.

2. Effect of Light Intensity and Temperature on Pigment Production

Germination of the seed was carried out in the same manner as for the light quality studies except that they were transferred to 3 inch peat pots instead of the plastic pots, and 24 seeds were used per pot instead of 15. After the seedlings were transferred to the growth chambers, they were developed for four cycles of 16 hours light and 8 hours darkness. Harvesting, drying and storage procedures were the same as in the light quality experiments. Four samples were harvested from each replication and the entire study was replicated after a time lapse.

3. Effect of Various Chemicals on Pigment Production

The germination was the same as in the above studies for the first 24 hours. After selection for uniformity, 10 seedlings were planted in vermiculite contained in small glass jars. These were then wetted with 60 ml of the solutions of chemicals to be tested (except for

treatment number 15). The volumes were such that the vermiculite was flooded nearly to the level of the planted seeds. The chemicals and the concentrations used are listed in table 12. (P. 59).

After wetting, the jars were immediately placed in the growth chamber. The experiment was arranged in a randomized block design with 4 replicates. The light cycle was 16 hours light and 8 hours darkness. On the second and the fifth days after placing in the growth chamber the liquid level was brought to the surface of the vermiculite with distilled water.

The seedlings emerged 2 days after being placed in the growth cabinet. On the fifth day treatment number 15 was given to the seedlings by applying a water extract of mutant leaves to the whorl of the seedlings. This was done a second time after a 3 hour interval. The extract was made from the fresh leaves of 5-day-old mutant seedlings grown in the dark at 20°. The leaves were minced in the cold (4°) using a Waring blender and filtered through cheese cloth. The filtrate was centrifuged at 20,000 g for 10 minutes and the supernatant freeze-dried. An aliquot of the powder was made up in a concentration of .025 g/ml of water. This solution was then applied to the seedlings.

Also on the fifth day after placing in the growth cabinets, the seedlings in the other treatments were sprayed with the same chemical solutions with which they had been initially wetted. A few drops of a 1% detergent (Duponol C) solution was added to each to aid in wetting of the leaves.

After 7 days in the growth cabinet the primary leaves were harvested and handled as in the above pigment studies.

4. Amino Acid Investigations

Since it was desired that amino acid concentrations be expressed on the basis of dry seed weight for the various stages of seedling development, and since not all seeds could be expected to develop evenly enough to be included in a sample, each seed was weighed before germination was begun. A sample weight was therefore a sum of the individual weights of those seeds whose seedlings were finally selected on the basis of their uniformity.

After weighing the seeds were placed on trays of vermiculite which were just flooded with distilled water, and their positions recorded. The seeds were not covered with absorbent tissue in this study, and although they were pressed into the vermiculite so that they were even with the surface, they were not covered with it. The trays were then placed into the germinating cabinet at 24°. The seeds used in this study were produced from a different harvest than those used in the pigment studies and germination was not so rapid in this second seed lot. Therefore seedling selection was not made 24 hours after starting germination as with the other material. Selection of seedlings was carried out prior to harvest under a green safe-light.

In the first experiment etiolated seedlings were harvested after 1, 2, 4, 5, 7, and 9 days of development. In the second experiment the seedlings were developed for 5 days under etiolated conditions

at 24° and then transferred to 1500 ft-c of light and 15.5° or 33.5° . Two cycles of 16 hours light and 8 hours darkness were given and the plants harvested after the last dark period — at the end of day 7. Material which was protected from the light was also transferred to 15.5° and 33.5° on day 5 for use as controls.

In the first experiment entire seedlings were used for analysis. In the second experiment the seedlings were divided into shoot, and root plus endosperm. The fresh weight of the leaf material was taken in the latter experiment. In harvesting the etiolated material, no effort was made to completely exclude white light. However, subdued light was used and harvesting was carried out as quickly as possible. Thus exposure was limited to dim light for periods of 5 to 20 minutes before the material was killed. Boiling 95% ethanol was used for killing and the samples were stored in this solvent until complete extraction was carried out.

4. Ribonucleic Acid Measurements

The seeds were planted in 6 inch plastic pots containing California mix, wetted with tap water, and placed directly into the growth chamber. The temperature was 20° , the humidity 50% and the light intensity 1500 ft-c. The light cycle was 16 hours light and 8 hours darkness. The pots were examined each day to eliminate early- and late-emerging seedlings. The age of the seedlings was dated from the time of emergence, which was about 3 days after wetting. Primary leaves were harvested on the third, fifth and twelfth days. The leaf material was freeze-dried, ground in a Wiley mill to pass

a 60 mesh screen, and stored in a dessicator over P_2O_5 at -28° until analyzed.

IV. Extraction and Sample Preparation

1. Leaf Pigments

The same procedure of extraction was used in all the studies concerning pigment production.

The dry leaf tissue was weighed and then ground using a mortar and pestle with fine sand as an abrasive. Complete extraction of the pigments was found to be dependent upon very thorough grinding. Pigments were extracted using 80% acetone. The suspension was transferred to a buchner funnel and filtered through a double layer of Whatman No. 1 filter paper. The samples were made to an appropriate volume and an aliquot used immediately to measure the pigment concentration.

2. Amino Acids

The tissues to be extracted were ground using a mortar and pestle. Fine sand was added as an abrasive and the 95% ethanol in which the tissues were killed and stored was used as the initial extracting solvent. The volume of ethanol for this first extraction was about 35 ml.

After thorough grinding the samples were rinsed into 50 ml steel centrifuge tubes and centrifuged at 20,000 g for 10 minutes. The supernatant was decanted and the pellet resuspended in 25 ml of

95% ethanol and let stand approximately 5 minutes before centrifuging. The supernatant was decanted and the pellet again suspended in 25 ml of ethanol, and handled as before. The 3 supernatants were tested with ninhydrin and the results indicated that practically all of the ninhydrin-positive material was extracted with the first 35 ml of ethanol. The third extract was only very slightly ninhydrin positive.

The three supernatants for each sample were combined and taken to dryness using a rotary flash-evaporator at room temperature. Five to 10 ml of water were then added to the flask. Lipid-soluble material was extracted from the flask with several aliquots of diethyl ether by adding this solvent directly to the flask, rinsing or shaking gently, and then siphoning off the ether fraction using a fine capillary tube attached to a vacuum flask. When the ether fraction was re-extracted with water, no ninhydrin positive material was reclaimed. In the case of leaf tissue which had been exposed to light, the ether extract was used to measure chlorophyll production in the leaves. (This chlorophyll analysis was carried out in the same manner as those already described except that the extinction coefficients of Comar and Zscheile (1942) were used for calculation of concentrations.)

The water solution which remained in the flask was flash evaporated to a small volume at room temperature and then freeze-dried. The sample was finally made up to volume by pipetting into the flask the desired amount of a 1:1 mixture of distilled water and a "sample diluting buffer". The final pH was 2.2. The composition

of the buffer is given in table 4. The amino acid in this extract was taken as the "free" amino acid of the plant tissue. Not included in this analysis were any peptides which were extracted with 95% ethanol.

The pellets which were left after the ethanol extraction were suspended in diethyl ether, centrifuged, and the ether decanted and discarded. The pellets were air dried and then hydrolyzed for 24 hours with constant boiling HCl. The digests were flash evaporated to dryness at approximately 80°. Ten ml of water were added to the flask and the pH brought to 2-4 using KOH. Ten ml of the sample diluting buffer were added and the sample made to a suitable volume with a 1:1 water:sample-diluting buffer. The final pH was 2.2. The volume to which the samples needed to be made for analyses was not only dependent upon the number of seeds or seedlings used, but also on the stage of development and the conditions under which they were grown.

3. Ribonucleic Acid

a. Phenol Extraction Method

The extraction procedure was similar to that carried out by Colter and Brown (1956), but only the ribosomal RNA (rRNA) was analyzed.

One g of 12 day old, and 1.5 g of 5 day old dry primary leaf tissue was ground with fine sand using a mortar and pestle. The tissue was transferred to 200 ml centrifuge bottles and 25 ml

of saturated phenol and 25 ml of .05 M phosphate buffer, pH 6.8, were added. The mixture was shaken vigorously for 25 minutes at room temperature.

The suspension was then centrifuged at 11,500 g and the aqueous layer siphoned off. The last bit of this layer, contaminated with the phenol layer, was siphoned into a 15 ml heavy-walled glass centrifuge tube and recentrifuged at 20,000 g. The aqueous layers were combined in a 50 ml graduated conical tube and then washed 3 times with an equal volume of diethyl ether in order to remove most of the phenol from the aqueous phase. The solution was placed under a gentle air flow to remove the ether. The extract was made to approximately 1 M with NaCl and stored at 4° for 24 hours.

The precipitate which formed was centrifuged at 8500 g and the supernatant discarded. The pellet was washed by suspending and centrifuging twice in 15 ml portions of the following solvents: (i) 67% ethanol, (ii) 95% ethanol, and (iii) diethyl ether. These washings removed the last traces of phenol. The pellet was dried using a gentle air flow and then weighed.

In the case of the 12 day old leaf all the dried pellet was used for analysis. In the 5 day old leaf extract an aliquot of 6 mg was used. The powder was dissolved in 300 λ of water and a 10 λ sample diluted to 10 ml to check the purity of the RNA. To the remaining 290 λ there was added 30 λ of 10N NaOH, and the solution held at 22° for 40 hours.

At the end of the hydrolysis period 30 λ of glacial acetic acid was added, and in the case of the 12 day leaf extract, 100 λ of water. These solutions were then used to measure the nucleotide composition.

b. Modified Schmidt-Thannhauser (1945) Method for Total RNA

Preliminary extraction of the leaf material was patterned after that of Ogur and Rosen (1950) and Nieman and Poulsen (1963).

One gram samples of dry leaf tissue were ground with a mortar and pestle and rinsed into 50 ml steel centrifuge tubes with 40 ml of boiling 95% ethanol and boiled for 2 minutes. They were centrifuged at 20,000 g for 10 minutes and the supernatant discarded. Each pellet was then extracted by suspending and centrifuging using 40 ml portions of the following reagents: (i) 95% ethanol at room temperature — once; (ii) 50% ethanol adjusted to pH 4.5 with glacial acetic acid — 3 times; (iii) Cold (4 $^{\circ}$) .2N perchloric acid (PCA), held 15 minutes before centrifuging — twice; (iv) 95% ethanol at room temperature — once; (v) Boiling 95% ethanol : diethyl ether (3:1), held 3 minutes before centrifuging — twice; (vi) diethyl ether at room temperature — once. The pellet was then air dried to remove traces of ether.

The dry tissue was suspended in 15 ml of 1N KOH and hydrolyzed for 18 hours at 35 $^{\circ}$. The solution was then made to pH 4.5 with 7N PCA and the sediment centrifuged off at 20,000 g for 15 minutes. The supernatant was decanted and the pellet washed 3 times with 8 ml portions of .2N PCA. The supernatants were combined and carefully

neutralized with KOH. The precipitate was centrifuged off at 20,000 g for 20 minutes and the supernatant flash-evaporated at 40°. The residue was made to 10-12 ml with water and stored at 4° for 18 hours. The precipitate which formed was centrifuged off at 20,000 g for 20 minutes and the supernatant then decanted and freeze-dried. The powder was collected and weighed, and 150 mg aliquots dissolved in 2 ml portions of water in 15 ml conical centrifuge tubes. If insoluble material was present, it was centrifuged off at 10,000 g. The supernatant was then used for estimation of nucleotide concentrations.

V. Measurement

1. Leaf Pigments

Measurement was carried out employing a Beckman DK-1 spectrophotometer. The optical density of the extracts was routinely measured at 663, 645, and 440 mμ, these being the respective peak absorption wavelengths for chlorophyll a, chlorophyll b and carotenoids in acetone. The latter value is somewhat arbitrary depending upon the exact ratios of the pigments in the mixture. The major carotenoids, however, have their maximum absorption coefficients near to 440 mμ.

The concentrations of chlorophyll a and b were calculated using the equations given by Maclachlan and Zalik (1963).

These were:

$$C_a = \frac{(12.3 D_{663} - .86 D_{645})}{d \times 1000 w} V$$

$$C_b = \frac{(19.3 D_{645} - 3.6 D_{663}) V}{d \times 1000 w}$$

where: C = concentration in mg/g leaf tissue; a = chlorophyll a;
b = chlorophyll b; D = optical density at the wavelength given in
mμ; V = volume of the sample in ml; d = path length of the cell in
cm; and w = weight of the sample in g.

The following equation, after that given by von Wettstein
(1957), was used to calculate the carotenoid concentrations.

$$C_c = \frac{[4.695 D_{440} - .268 (C_a + C_b)] V}{d \times 1000 w}$$

where: C_c = concentration of carotenoids in mg/g leaf tissue; C_a and
C_b are the concentrations of chlorophyll a and b in mg/l of extract;
and the other figures are the same as those in the preceding equations.

2. Amino Acids

For the analysis of the acidic and neutral amino acids,
one ml aliquots of the samples were pipetted onto a 50 cm column of
Beckman type AA-15 cation exchange resin held at 55^o, and forced in
with N₂ gas pressure. The amino acids were eluted from the resin
with sodium citrate buffer, pH 3.25, pumped at the rate of 68 ml/hour
for a period of 20 hours, followed by pH 4.5 buffer for 1.5 hours.
The basic amino acids were chromatographed on a 10 cm column of
Beckman type AA-27 resin at the above temperature and flow rate,
using citrate buffer at pH 5.28. The composition of these buffers
is given in table 4. Also 1 ml of 0.04M NaCN was added to each litre
of eluting buffer just prior to use.

The eluent containing the amino acids was mixed with ninhydrin reagent for color development. This reagent, pumped at the rate of 34 ml/hour, was composed of: 192.20 sodium propionate, 46.5 ml propionic acid, 20 g ninhydrin, 750 ml methyl cellosolve, and water to one liter. The color development was carried out at 100° for approximately 7 minutes. The optical density of the solution was measured at 440 and 570 mμ and recorded on a Honeywell 3 channel, dot-type recorder. The areas under the curves were measured by the height x width method which was calibrated using a standard mixture containing .5 μ moles/ml of each of the amino acids.

Table 4

Composition of Buffers used for Amino Acid Analysis

	Sample diluting buffer	Eluent buffers		
pH	2.2	3.25	4.25	5.28
Na Conc.	.20 N	.20 N	.20 N	.35 N
Citric acid	21 g (.11 N)	840 g	840 g	491 g
NaOH (97%)	8.4 g	330 g	330 g	288 g
Conc. HCl	16 ml	426 ml	188 ml	136 ml
Caprylic acid	.1 ml	4.0 ml	4.0 ml	2.0 ml
Thiodiglycol	20.0 ml	200 ml		
Brij-35	2.0 ml	2.0 ml	80 ml	40 ml
Final vol.	1 l.	40 l.	40 l.	20 l.

Since the concentration of the different amino acids in the extracts varied so widely, difficulty was encountered in the quantitative measurement of all amino acids present using a single analysis. Consequently there are cases where certain amino acids were identified by their position in the eluent, but no estimation of their concentrations was possible. However, in the analysis procedure a factor of 20 times can be quite easily tolerated in the relative concentrations of the amino acids.

The analysis of the free amide concentrations in the extracts was attempted using two different methods.

In the first method the procedure used for the assay of acidic amino acids was followed until aspartic acid was begun to be eluted and recorded on the chart. The entire eluent flow was then directed to a collector and 25 one minute fractions taken. Since on the AA-15 resin at 55° the asparagine and glutamine overlap threonine and serine, one drop was taken from each tube and tested with ninhydrin to locate this group. These fractions were then combined and evaporated to dryness in 250 ml flash evaporation flasks. The residue was rinsed into a 10 ml test tube using approximately 6 ml of water which was subsequently removed by freeze-drying.

The amides were hydrolyzed to the respective acids by adding 1 ml of 1N HCl and refluxing for 3 hours. The acid was removed by freeze-drying and the sample made to 3 or 5 ml with sample diluting buffer. One ml of this solution was chromatographed on the AA-15 resin and the values of aspartic and glutamic taken as the concentration of the amides in the original eluent.

The second procedure for amide analysis involved the measurement of aspartic and glutamic acid contents before and after hydrolysis of the extracts with 1N HCl for 3 hours, and taking the differences as the respective amide concentrations. This was done by pipetting 0.5 ml of extract and 0.5 ml of 2N HCl into a 10 ml test tube and refluxing at 100°. The acid was then neutralized, the solution freeze-dried, and the sample made up to 3 or 5 ml with sample diluting buffer. One or 2 ml of this was chromatographed and the results compared to those previously obtained on the unhydrolyzed sample.

Using either of the procedures for amide analysis, the content of free threonine and serine could be determined.

3. Ribonucleic Acid (RNA)

The following technique is essentially that which was kindly supplied by Dr. Lane of the Biochemistry Department, University of Alberta, Edmonton.

The purity of the powdered extracts was determined by assuming an extinction coefficient of 210 for a 1% solution of RNA at 260 mμ ($E_{260}^{1\%}$). Therefore:

$$\% \text{ purity} = \frac{OD_{260} \times \text{dilution factor} \times 10}{210 \times \text{wt of powder used in mg}} \times 100$$

Where OD_{260} is the optical density of a solution of the RNA, the purity of which is being determined.

The separation of the nucleotides contained in the RNA hydrolysates was carried out using a paper chromatographic technique.

For this procedure Whatman No. 1 paper was impregnated with ammonium sulfate by spraying the paper with a one-tenth saturated solution and allowing the excess liquid to drip off.

Depending upon the purity of the extract, 20 to 80 λ were spotted on the paper in a band approximately .7 x 2.5 cm using 20 λ aliquots and drying with a hair dryer. The chromatograms were developed for 22 - 26 hours by the descending method using 75% ethanol in a 12 x 12 x 24 inch, all glass tank. Two 250 beakers containing about 150 ml of the developing solvent were placed at the bottom of the tank, but no wicks were used to further saturate the atmosphere.

When fully developed, the chromatograms were removed from the tank, air dried, and the nucleotides located by scanning with a short wave, Mineralight (model UVS 11, Ultra-violet Products, Inc., San Gabriel, Cal.) which proved to be very efficient for this purpose. The spots were traced and then cut out along with a blank taken adjacent to, and of the same size as each spot. These paper discs were then cut into small bits, placed in a 15 ml conical centrifuge tube and 3 or 4 ml of .12N HCl added. The tubes were placed on a vortex mixer until the paper pieces were fairly well disintegrated. The paper was centrifuged off at 10,000 g for 5 minutes and the optical density of the supernatant measured between 300 and 230 m μ in the Beckman DK-1 spectrophotometer.

The concentration of the nucleotides were calculated using the following molar extinction coefficients at 260 m μ (E_{260}) in acid:

$$\text{Cytidylic acid} = 6.8 \times 10^3$$

$$\text{Guanylic acid} = 11.8 \times 10^3$$

$$\text{Adenylic acid} = 14.2 \times 10^3$$

$$\text{Uridylic acid} = 10.0 \times 10^3$$

$$\text{Thus: } \mu \text{ moles Np per analysis} = \frac{\text{OD}_{260} \times \text{Vol in ml of solution read} \times 1000}{E_{260}}$$

$$\text{and } \mu \text{g Np/g leaf tissue} = \frac{\mu \text{ moles Np per analysis} \times \text{mol. wt.} \times \text{dilution factor}}{\text{wt of leaf tissue}}$$

RESULTS AND DISCUSSION

I. Pigment Determinations

1. Light Quality

The differences between the mutant and normal seedlings in accumulation of chlorophyll were highly significant at all wavelengths used. Normal seedlings grown in the green and red light treatments did not differ in chlorophyll content, but had significantly more chlorophyll than the other treatments. In the mutant there was significantly more chlorophyll in the green light than in any other treatment, including the red. Thus the mutant was less efficient at accumulation of chlorophyll in red light than was normal barley. Both lines acted the same in producing markedly less chlorophyll in the red-far-red than in any other treatment (table 5, fig. 1).

That there was a differential response in chlorophyll accumulation by the normal and mutant lines to light of different quality, was borne out by the highly significant interaction of lines x light quality in the analysis of variance (table 6). The calculation of chlorophyll content as a per cent of the control clearly indicated that the major difference occurred in the green, yellow and red treatments. Under green light the mutant was relatively more efficient than the normal, whereas under yellow and red the reverse was true.

There was little difference in the ratios of chlorophyll a to b, which for the normal ranged from 2.4 in the red to 2.8 in the

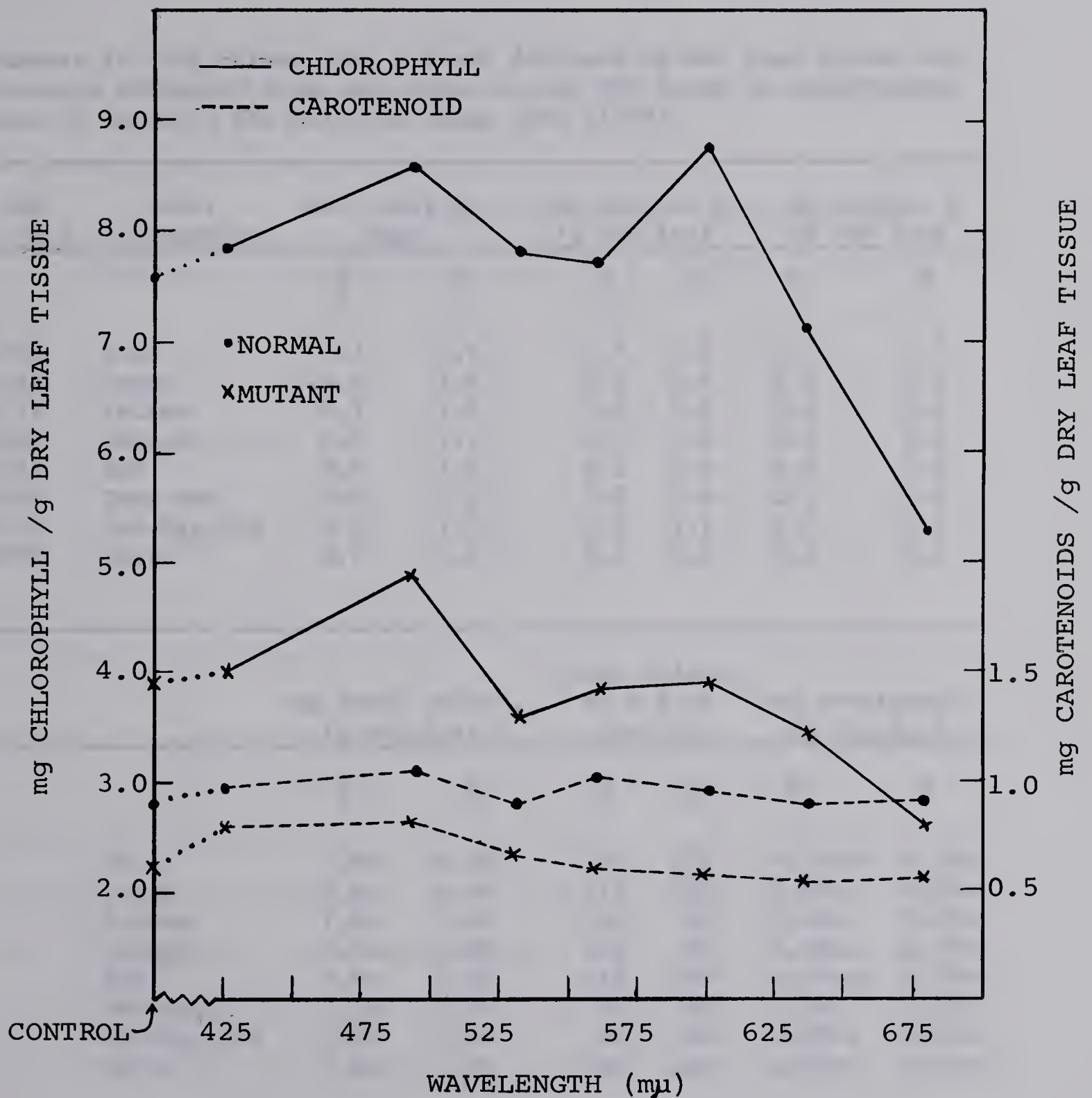


Fig. 1. Effect of light quality on the pigment content of normal and mutant barley seedlings. Each point on the graph represents the mean of 6 readings.

Table 5

Pigment Concentrations of Normal and Mutant Barley Seedlings Exposed to
5 Cycles of Light of Several Spectral Bands

Numbers in each column that are not followed by the same letter are significantly different from each other at the 95% level of significance as judged by Duncan's New Multiple Range Test (1955).

λ at 50% trans. (mu)	Color designation	Ave. leaf wt. (mg)		mg chloro. <u>a</u> /g dry leaf		mg chloro. <u>b</u> /g dry leaf	
		<u>N</u>	<u>M</u>	<u>N</u>	<u>M</u>	<u>N</u>	<u>M</u>
393-463	Blue	8.3	8.0	5.7	3.0	2.2	1.0
406-532	Green	8.2	7.6	6.3	3.6	2.3	1.3
500-577	Yellow	8.3	7.4	5.6	2.6	2.2	0.9
540-605	Orange	8.5	7.7	5.7	2.8	2.0	1.0
580-655	Red	8.4	7.8	6.2	2.8	2.6	1.0
610-705	Deep-Red	8.5	7.7	5.0	2.4	2.1	1.0
643-738	Red-Far-Red	8.7	7.5	3.9	1.9	1.4	0.8
346-890	White*	8.7	7.3	5.4	2.8	2.2	1.1

	mg total chloro. /g dry leaf		Total chloro. as a % of control		mg carotenoids /g dry leaf	
	<u>N</u>	<u>M</u>	<u>N</u>	<u>M</u>	<u>N</u>	<u>M</u>
Blue	7.8b	4.0b	103	103	0.97abc	0.80a
Green	8.6a	4.9a	113	125	1.04a	0.82a
Yellow	7.8b	3.6b	102	92	0.88c	0.66b
Orange	7.7bc	3.8b	101	98	1.01ab	0.59bc
Red	8.8a	3.9b	115	100	0.95abc	0.58bc
Deep-Red	7.1c	3.5b	93	89	0.89c	0.53c
Red-Far-Red	5.3d	2.6c	69	66	0.90bc	0.57bc
White*	7.6bc	3.9b	100	100	0.92bc	0.61bc

* Control — incandescent light.

green, and for the mutant ranged from 2.4 in the red-far-red to 2.9 in the blue.

Table 6

Analysis of Variance for Pigment Concentration

Source	df	<u>Total Chlorophyll</u>		<u>Carotenoid</u>	
		ms	f	ms	f
Lines	1	351.67	313.99 ^{**}	2.193	95.35 ^{**}
Light quality	7	8.25	7.37 ^{**}	.067	2.91
Lines x light quality	7	1.12	4.31 ^{**}	.023	2.55 [*]
Error	78	.26		.009	

* Significant at the 95% level

** Significant at the 99% level

In all treatments the normal seedlings accumulated significantly more carotenoids than did the mutant. In the normal there was no significant difference in the carotenoid concentration between the blue, green, orange and red treatments. Only green was superior to white light while yellow and deep-red were inferior. In the mutant, however, both blue and green light stimulated significantly more carotenoid accumulation than did any other treatment, and only deep-red was inferior to the control (table 5). The significant interaction of lines x light quality for carotenoid production (table 6) again indicates that the two barley lines respond differently to light quality.

In general, light of shorter wavelengths appears to be more beneficial to the mutant than to the normal, the reverse being true for longer wavelengths. This differential response suggests that the mutation has affected the absorbing unit in some manner so as to either change the absorption characteristics, or to change the manner in which the light energy is utilized after absorption.

2. Light Intensity and Temperature Studies

The data (table 7) indicates that the same conditions of light intensity and temperature used in this study were optimal for chlorophyll production in both the mutant and the normal lines. These conditions, on the basis of chlorophyll per unit dry weight, were 24.5° and 1020 ft-c, and on the basis of chlorophyll per cm of leaf length, 24.5° and 1800 ft-c. However, the relative efficiency of the two lines differed considerably under other conditions as shown in table 8. It is evident that the mutant was affected more strongly than the normal by low temperatures at all intensities. The Q_{10} values for the mutant varied from 3.1 to 4.4 over the temperature range 14.0 to 19.5, whereas comparable values for the normal were 1.0 to 1.4 (fig. 2). This differential effect of temperature on the two lines was confirmed by the highly significant interaction of lines x temperature in the analysis of variance (table 9).

Figure 2 presents the data on total chlorophyll accumulation under the different light intensities and temperatures used. Under all conditions there was significantly more chlorophyll in the normal than in the mutant. Under 2600 ft-c temperature had little influence

Table 7

Influence of Light Intensity and Temperature on the Total
Chlorophyll Accumulation in Normal and Mutant
Gateway Barley Seedlings

		mg Chlorophyll/g Dry Leaf Tissue			
		Temperature*			
Light Intensity		14.0	19.5	24.5	29.0
Normal	320	10.20 \pm 3	12.49 \pm 2	12.58 \pm 2	11.76 \pm 2
	1020	10.05 \pm 3	11.87 \pm 1	14.17 \pm 1	11.58 \pm 3
	1800	8.10 \pm 1	8.59 \pm 2	10.85 \pm 2	10.00 \pm 3
	2600	9.12 \pm 2	9.03 \pm 4	9.26 \pm 2	9.00 \pm 3
Mutant	320	2.53 \pm 5	5.45 \pm 3	6.95 \pm 4	6.41 \pm 1
	1020	2.19 \pm 3	6.30 \pm 2	7.84 \pm 2	6.74 \pm 5
	1800	1.70 \pm 2	4.18 \pm 7	7.08 \pm 2	6.50 \pm 5
	2600	3.09 \pm 4	5.16 \pm 2	6.36 \pm 5	6.70 \pm 1
		Calculated as μ g Chlorophyll/cm Leaf Length			
Normal	320	9.4	10.2	11.0	9.7
	1020	10.1	10.7	13.1	10.6
	1800	9.3	9.5	13.4	8.9
	2600	11.4	9.9	11.4	8.8
Mutant	320	2.3	4.3	5.2	5.0
	1020	2.0	5.2	6.4	5.6
	1800	1.4	3.7	6.8	5.7
	2600	2.7	4.5	5.8	5.7

* Standard errors are expressed as a % of the mean.

Table 8

The Relative Efficiency of Total Chlorophyll Production in Mutant and Normal Barley Seedlings under Different Conditions of Light Intensity and Temperature

Light Intensity	$\frac{\text{Chlorophyll per cm leaf in Mutant}}{\text{Chlorophyll per cm leaf in Normal}} \times 100$			
	Temperatures			
	14.0	19.5	24.5	29.0
320	24	42	47	51
1020	20	49	49	53
1800	15	39	51	64
2600	25	46	51	65

Table 9

Influence of Light Intensity and Temperature.

Analysis of Variance for mg Total Chlorophyll/g Dry Tissue

Source	df	ms	f
Lines (L)	1	1742.85	157.65 ^{**†}
Temperatures (T)	3	144.80	21.58 ^{**†}
Light Intensity (I)	3	50.49	7.56 ^{**†}
L x T	3	26.33	27.80 ^{**}
L x I	3	26.10	27.56 ^{**}
T x I	9	7.04	7.43 ^{**}
L x T x I	9	0.947	2.53 ^{**}
Error	214	0.374	

^{**} Significant at the 99% level.

[†] Tested against a composite error.

on chlorophyll accumulation in normal barley seedlings. It is also evident that in general light intensity had a stronger effect on the normal than on the mutant. The highly significant F value for the interaction of lines x light intensity confirmed this.

The rather high chlorophyll accumulation observed in both lines under 2600 ft-c at 24⁰ may have been caused by an increase in the leaf temperature above the recorded temperature. It is generally recognized that leaves of plants may, under conditions of high light intensity, attain a temperature somewhat higher than that of the surrounding air (Curtis and Clark, 1950). This may have occurred in spite of the 13 cm water filter. Results at lower intensities, especially for the mutant, show that a small increase in temperature would result in a sharp increase in chlorophyll content.

Disregarding the high intensity result, the trend at 14.0⁰ was toward greater efficiency in the mutant relative to the normal as light intensity decreased (table 8). At 24.5⁰ there was little effect of light intensity, whereas at 29.0⁰ the low temperature trend was reversed so that the relative efficiency of the mutant increased with increased light intensity. This shift in the accumulation of chlorophyll in the mutant relative to the normal was significant as shown by the triple interaction in the analysis of variance in table 9.

Little difference existed in the ratio of chlorophyll a to b. The ratios for both lines varied from 2.4 to 2.9 in all cases except for the mutant at low temperature, where the ratio reached

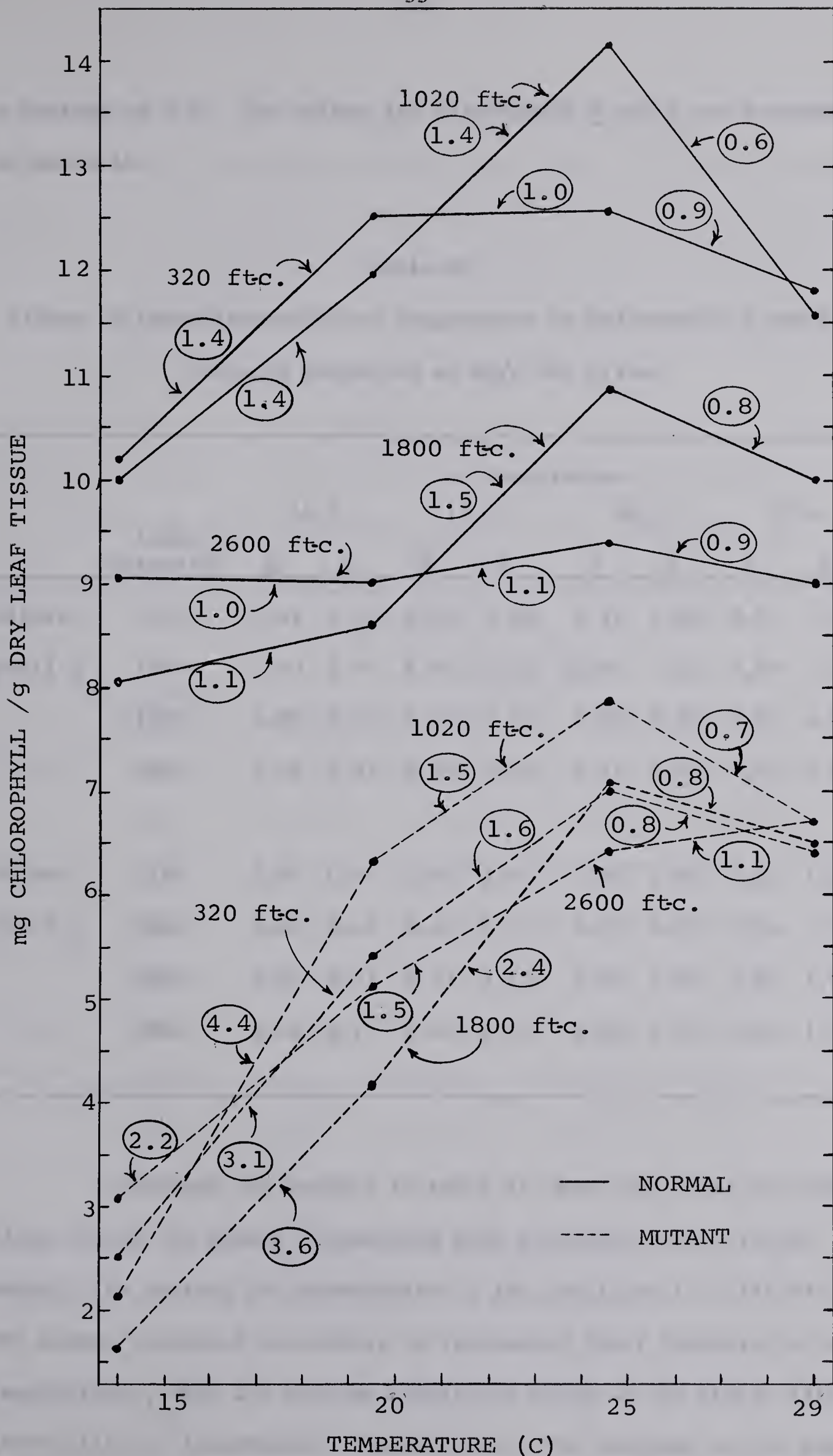


Fig. 2 The effect of temperature on chlorophyll production at various light intensities. Each point represents the mean of 8 samples. The approximate Q_{10} values are encircled.

a maximum of 3.9. The values for chlorophyll a and b are presented in table 10.

Table 10

Effect of Light Intensity and Temperature on Chlorophyll a and b
Contents Expressed as mg/g Dry Tissue

		Temperature							
		14.0		19.5		24.5		29.0	
	Light Intensity	N	M	N	M	N	M	N	M
Chloro-	320	7.41	1.92	9.02	3.86	9.10	4.92	8.34	4.54
phyll <u>a</u>	1020	7.40	1.74	8.66	4.58	10.04	5.56	8.56	4.77
	1800	6.08	1.30	6.35	3.07	7.88	5.09	7.14	4.64
	2600	6.72	2.35	6.60	3.74	6.77	4.64	6.44	4.72
Chloro-	320	2.82	0.61	3.46	1.60	3.48	2.02	3.42	1.86
phyll <u>b</u>	1020	2.70	0.46	3.24	1.72	4.13	2.24	3.34	1.98
	1800	2.02	0.41	2.23	1.12	2.98	1.99	2.84	1.86
	2600	2.40	0.74	2.44	1.43	2.50	1.73	2.56	1.99

Although the results in table 11 show that under all conditions tested the normal accumulated more carotenoid than did the mutant, the pattern of accumulation in the two lines is quite different. The mutant responded favourably to increasing light intensity at all temperatures, with the optimum conditions being at the higher light intensities at intermediate temperatures. The response in the normal

was somewhat more variable and optimum conditions were at lower light intensities at intermediate temperatures. Thus, although an analysis of variance was not carried out on these data, it appears obvious that carotenoid accumulation in the mutant differs from the normal in response to changing conditions of light intensity and temperature.

Table 11

Influence of Light Intensity and Temperature on the Carotenoid Content of Mutant and Normal Gateway Barley Seedlings

		mg Carotenoid/g Dry Tissue [*]			
		Temperatures			
Light Intensity		14.0	19.5	24.5	29.0
Normal	320	1.41 \pm 14	1.88 \pm 4	1.82 \pm 5	1.69 \pm 3
	1020	1.52 \pm 13	1.81 \pm 2	1.89 \pm 7	1.68 \pm 4
	1800	1.56 \pm 10	1.85 \pm 4	1.66 \pm 5	1.69 \pm 4
	2600	1.72 \pm 9	1.69 \pm 7	1.52 \pm 5	1.49 \pm 3
Mutant	320	.91 \pm 10	.95 \pm 7	1.03 \pm 4	1.04 \pm 3
	1020	.94 \pm 9	1.22 \pm 4	1.30 \pm 2	1.13 \pm 5
	1800	1.00 \pm 8	1.28 \pm 4	1.41 \pm 4	1.33 \pm 5
	2600	1.22 \pm 6	1.42 \pm 4	1.44 \pm 2	1.26 \pm 2

* Standard errors are expressed as a % of the mean.

3. The Effect of Various Chemical Treatments

The mean values for pigment production are given in table 12. Those marked with an asterisk differ significantly from the control as judged by Dunnett's test (1955) (d' values are given). For ease of comparison the data has also been expressed as a per cent of the control.

Under the conditions in which the study was carried out there was no significant difference between the chlorophyll accumulation of the barley lines (table 13), but there was a highly significant difference in carotenoid production.

The important results in this experiment are those in which the mutant and normal responded differently to the chemical treatment. In this respect N⁶-benzyladenine and CCC were outstanding. In the case of N⁶-benzyladenine both the chlorophyll and carotenoid content of the normal was reduced by a significant amount whereas that of the mutant was virtually unchanged. The same trend is seen in the effect of kinetin, which is a close relative of N⁶-benzyladenine, and although the effect was not significant at the 95% level, it is possible that with a higher concentration it would have been. Benzimidazole, a compound somewhat similar to kinetin and N⁶-benzyladenine, had a drastic effect on pigment production in both lines. The concentration used was apparently too high to detect, with any certainty, a differential effect on the two lines, although the chlorophyll in the mutant was reduced by 78% whereas in the normal the reduction was by only 57%.

Table 12

The Effect of Various Chemicals on Pigment Accumulation in Normal and Mutant Barley Seedlings

No.	Chemical treatment	Conc. (ppm)	Total Chlorophyll		As % of control		Carotenoids	
			mg/g dry leaf				mg/g dry leaf	
			<u>N</u>	<u>M</u>	<u>N</u>	<u>M</u>	<u>N</u>	<u>M</u>
1	H ₂ O		6.92	6.31	100	100	1.15	1.26
2	Fe (ic)	100	6.69	6.48	97	103	1.08	1.26
3	Mg.	1000	7.45	6.63	108	105	1.09	1.23
4	Co,B,Cu	10,10,10	2.88*	0.79*	42	13	0.81*	0.61*
5	Gibberellic acid	.5	4.50*	4.30*	65	68	0.73*	0.89*
6	Kinetin	.1	6.14	6.56	89	104	0.95	1.26
7	CCC	500	7.38	8.52*	107	135	1.15	1.53*
8	N ⁶ -benzyladenine	.1	4.81*	6.40	70	101	0.71*	1.08
9	Gramine	1000	8.50*	7.25	123	115	1.16	1.37
10	Glycyl-glycine	1500	7.32	5.94	106	94	1.07	1.20
11	Benzimidazole	200	2.96*	1.12*	43	18	0.49*	0.29*
12	Glutathione	200	5.89	6.22	85	99	0.92*	1.28
13	Asparagine	1500	5.41*	5.83	78	92	0.76*	1.18
14	Glutamine	1500	5.79	5.02	84	80	0.90*	1.11
15	Mutant water extract	25,000	6.34	5.42	92	86	0.96	1.22
	d'		1.22	1.61			0.22	0.25

* differ from the control at the 95% level of significance as judged by Dunnett's test (d').

Table 13

Analysis of Variance for the Data Concerning the Effect
of Various Chemicals on Total Chlorophyll
and Carotenoid Accumulation

Source	df	Chlorophyll		Carotenoid	
		ms	F	ms	F
Treatments	3	25.55	11.56**	.472	6.79**
Lines	1	5.13	2.32	1.072	15.40**
Lines x Treatments	14	2.21	4.70**	.0696	5.59**
Error	87	.47		.0124	

** Significant at the 99% level.

The effect of CCC on pigment production in the mutant was particularly beneficial. Although the chlorophyll content of the normal was increased somewhat, this was not nearly significant and the carotenoid was not increased at all. A difference in sensitivity to CCC has also been shown using different plant species (Norris, 1964). There was no reduction in the length of the seedlings due to CCC treatment in this test, presumably because of the short term of the experiment. In a preliminary test using this chemical in the same concentration, but beginning germination directly in the solution, a reduction of 60% was noted in the height of both the normal and mutant seedlings after 16 days of development.

Gramine appears to increase the chlorophyll content of both barley lines, and although the increase in the mutant is not significant at the 95% level, a differential action of this chemical is questionable.

The only other treatment which indicated a substantially different action on the two lines was asparagine. This treatment significantly reduced pigment content in the normal but not in the mutant. This result is somewhat surprising in view of the high asparagine contents of mutant seedlings compared to those of normal as will be discussed in a later section.

It is perhaps worth noting that all of the chemicals which showed a differential action on the two barley lines were nitrogen-containing compounds, and further that 3 of these - kinetin, N⁶-benzyladenine and gramine - have a similar type ring structure as a nucleus. Of additional significance is the fact that in nearly every case the action of the chemicals was the same on both chlorophyll and carotenoid production, suggesting that the effect of the chemicals was not directed at pigment molecule synthesis but toward some factor which affects accumulation of all the pigments measured.

Although some of the other chemicals used had an effect on pigment production, their effects were similar on the normal and mutant and are therefore of little importance in the determination of the mutant gene action.

II. The Amino Acid Composition of Seedlings

The amino acid values given in the following tables were generally based on 3 samples although in some cases only duplicates were used. In most instances the agreement between values was good, the standard error of measurement being generally less than 5% of the mean for protein hydrolyzates and 10% for free amino acids. In a few cases, particularly in the measurements of free acids present in very low concentration, the standard error exceeded the above limits.

Throughout the experiments, reliable measurements of certain amino acids were unattainable due to very low concentrations in the extracts. Free cysteine, methionine, and at some stages, proline, were the acids generally present in lowest concentration. In the acid hydrolyzates of the pellets the instability of cysteine and methionine (in addition to tryptophan) rendered accurate measurement of these impossible. In addition, the inability to consistently separate the basic amino acids of unhydrolyzed samples by the technique employed resulted in the loss of these results in most cases. Consequently no data are to be presented for the basic amino acids in the free state. The few analyses that were successful indicated that very small amounts of lysine and arginine, but substantial amounts of histidine occurred in the free state. Only the results using the second method of amide measurement are to be given, since recovery of glutamine after chromatography was approximately 70%. The loss was probably due to conversion of glutamine to pyrrolidone carboxylic acid during chromatography as pointed out by Cocking and Yemm (1961) to take place on resins of the type used here.

Large amounts of aspartic acid were present in all of the extracts of hydrolyzed pellets from both experiments. In view of the proportion of barley protein made up of aspartic acid as reported by other workers (Yemm and Folkes, 1958) the amount of aspartic in the pellets was too great for it all to be incorporated into protein. The ammonia results indicated that substantial amounts of the aspartic residues were amidated. In view of the nearly ninhydrin negative results of the third extraction (see procedure for extraction) it cannot be concluded that a variable amount of asparagine was extracted in the procedure used. Also, fairly precise results for free asparagine, and aspartic acid in the pellets were obtained. For example, the highest concentration of asparagine was found to be 204.45 μ moles/g seed in the 9-day-old normal seedlings in the first experiment (table 14), and the standard error of this measurement was 6% of the mean. The highest content of aspartic acid in the pellets was 141.8 μ moles/g seed from the normal leaf grown at 33.5° in the dark (taken from table 16, p. 68). The standard error for this value was only 2.5% of the mean. At the lower concentrations of aspartic and asparagine there were cases where the standard error exceeded those mentioned above, but if variation was due to the process of extracting asparagine, it should be expected that precision and content would be inversely related, except at very low concentrations. An alternative explanation is that "free" asparagine is present in two forms; one which is extracted with 95% ethanol and one which is not. The difference between these forms could be in the binding to some other compound or perhaps only in the formation of the ring structure (Kretovich, 1959). At any rate the results from hydrolyzed pellets cannot be interpreted as representing

Table 14

Experiment 1

Free Amino Acids of Entire Seedlings at Various Stages
of Development in the Dark at 24.0°

		(μ moles/g seed)					
Age (days)		1	2	4	5	7	9
Aspartic	N	.09	.37	2.84	2.65	7.64	13.36
	M	.11	.35	2.44	2.08	8.79	10.36
Threonine	N	n	n	5.38	5.42	8.76	16.73
	M	n	n	6.87	5.90	9.40	15.39
Serine	N	n	n	5.43	5.84	9.38	20.49
	M	n	n	6.80	4.61	10.41	18.06
Glutamic	N	.22	1.26	6.90	6.34	11.08	13.92
	M	.36	1.17	5.94	4.66	11.45	15.11
Proline	N	2.28	4.58	14.36	9.03	6.39	1.54
	M	3.87	5.05	15.42	7.88	3.07	1.84
Glycine	N	.10	.42	2.03	2.59	2.94	5.16
	M	.17	.41	2.57	2.18	4.07	5.38
Alanine	N	1.00	2.38	6.83	11.10	14.46	22.50
	M	1.34	2.44	8.08	9.36	17.83	19.13
Valine	N	.44	2.57	14.22	16.96	18.34	25.72
	M	.67	2.32	15.77	16.96	23.63	27.63
Isoleucine	N	.24	1.71	9.74	10.77	11.66	12.91
	M	.39	1.54	10.06	11.61	13.45	13.29
Leucine	N	.47	3.20	14.52	12.61	10.98	8.14
	M	.71	2.85	15.63	13.31	11.02	8.34
Tyrosine	N	.14	.95	5.82	5.64	4.08	6.58
	M	.20	.92	6.59	4.98	4.64	6.29
Phenylalanine	N	.27	1.52	7.60	7.06	4.69	6.14
	M	.39	1.43	8.96	6.43	4.22	5.87
Asparagine	N	n	n	26.76	54.76	91.61	204.45
	M	n	n	32.57	41.24	102.48	168.93
Glutamine	N	n	n	48.60	32.04	24.20	15.42
	M	n	n	52.06	33.16	23.63	17.40

n = not determined

only protein composition insofar as aspartic acid is concerned. Nor can the results of "free" asparagine be considered as the total amount of asparagine which is present in the tissue but not bound in protein.

The results for the free amino acids and amides obtained in the first experiment, in which the total seedlings were used without any division into fractions, are given in table 14. It may be seen that little difference existed between the quantities extracted from the mutant and normal seedlings at any stage up to 9 days. The manner in which amino acids increased or decreased was quite different from one acid to another, but the two barley lines behave in the same way with respect to any particular acid. The free acids, aspartic, threonine, serine and glutamic, showed a decrease on the fifth with respect to the fourth day before continuing to accumulate as etiolation continued. Other free acids, namely glycine, alanine, valine, isoleucine and asparagine, increased steadily throughout the test period. Proline and leucine acted similarly in that they reached a maximum on the fourth day and then declined steadily. Free proline was present in very small amounts by the ninth day. A maximum was also reached by free tyrosine and phenylalanine on the fourth day, followed by a rapid decline to the seventh day, but these increased again on the ninth day. The maximum values for free glutamine were obtained on the fourth day — the first day tested; subsequently this amide declined in content as etiolation progressed. The results of this experiment are not at variance with any in the literature, although few direct comparisons can be made due to the use of different experimental conditions.

It is evident from the free amino acid results obtained in the second experiment in which light and temperature conditions were altered, that these environmental factors had a strong influence on the metabolism of the amino compounds. The results for the free amino acids, compiled for the entire seedlings, are given in table i of the appendix. The values were obtained by adding the results from the analyses of the free amino acids extracted from the root plus endosperm fraction to those obtained from the leaf fraction. The latter values are given in table 15. It is also evident from table 15 that light and temperature had a differential effect on the mutant and normal. At low temperature in the dark the mutant leaves accumulated greater amounts of free amino acids than did the normal. The values for the root plus endosperm were only slightly greater for the mutant than for the normal. At high temperature in the dark there was little difference between the free amino acid contents of the two lines. (In table 14 from the first experiment the results for the seventh day at 24.0° indicated that differences between the lines were intermediate to those from dark-treated seedlings in the second experiment.) These data suggest that the mutant is less efficient in its use of free amino acids for protein production, especially at low temperature. The results for the protein composition of leaf material (table 16) substantiates this suggestion. At 15.5° in the dark most of the amino acids in the mutant pellets were present at about 80% of the content found in the normal. (These data were also calculated on the basis of fresh leaf weight but are not given here since the relative values showed very nearly the same pattern as those calculated on the basis of seed weight.) The fact that lysine and arginine contents of the

Table 15

Experiment 2

Free Amino Acids from Leaves of Seedlings Grown 5 Days
in Darkness at 24.0°, Followed by 2 Days Under the
Conditions Indicated at the Top of Each Column

		(μ moles/g seed)			
		15.5°	33.5°	15.5°	33.5°
		dark	dark	light*	light*
Aspartic	N	3.32	3.09	3.46	3.69
	M	4.25	3.78	7.50	7.96
Threonine	N	3.34	7.59	2.53	2.78
	M	5.31	8.10	5.12	8.72
Serine	N	5.91	6.85	4.98	2.82
	M	8.07	8.04	9.52	7.51
Glutamic	N	6.89	8.53	8.11	7.54
	M	7.91	8.70	6.10	8.52
Glycine	N	1.34	2.33	1.46	.57
	M	2.17	3.02	2.10	1.80
Alanine	N	8.11	24.21	6.22	6.34
	M	11.41	24.29	8.08	13.09
Valine	N	7.08	16.85	3.86	2.05
	M	11.71	19.42	7.96	8.52
Isoleucine	N	3.77	7.99	2.14	.92
	M	6.75	8.52	5.06	3.26
Leucine	N	2.79	4.94	1.29	.78
	M	5.54	4.93	4.32	2.33
Tyrosine	N	.85	4.09	.68	.76
	M	1.55	4.43	1.00	1.72
Phenylalanine	N	1.05	2.60	.72	.64
	M	1.93	2.96	.65	.63
Asparagine	N	42.40	33.22	46.00	13.60
	M	60.00	36.07	74.94	84.09
Glutamine	N	1.60	.87	7.21	1.27
	M	7.20	1.32	23.01	5.68

* The light treatment was 16 hr light (1500 ft-c) and 8 hr darkness.

	<u>mg/g seed</u>
Total Chlorophyll Content of Mutant:	.087
Total Chlorophyll Content of Normal:	.606

Table 16

Experiment 2

Amino Acid Composition of Leaf Pellets of 7-day-old Barley Seedlings
Germinated 5 Days in Darkness at 24.0^o, Followed by 2 Days Under the
Conditions Indicated at the Top of Each Column
(μ moles/g seed)

	Normal				Mutant			
	15.5 ^o dark	33.5 ^o dark	15.5 ^o * light	33.5 ^o * light	15.5 ^o dark	33.5 ^o dark	15.5 ^o * light	33.5 ^o * light
Lys.	23.2	32.0	27.4	36.6	24.6	28.3	23.3	30.4
His.	7.7	8.9	7.6	11.2	6.8	7.9	6.0	9.1
NH ₄ ⁺	71.9	128.5	57.1	65.6	68.5	118.4	55.0	69.0
Arg.	15.6	19.9	18.4	23.7	15.8	17.2	14.5	21.0
Asp.	65.8	141.8	48.4	60.9	67.9	123.3	43.5	59.1
Thr.	23.2	25.6	23.1	33.9	18.2	20.7	17.6	26.5
Ser.	26.8	30.8	25.7	36.4	22.4	25.7	21.6	30.4
Glu.	47.3	49.7	43.5	62.2	35.2	39.7	35.0	48.5
Pro.	26.2	24.6	23.4	36.9	19.8	21.0	17.4	27.3
Gly.	40.5	44.7	42.7	63.0	32.7	37.3	32.5	47.0
Ala.	42.6	47.7	43.6	64.8	34.8	38.8	33.4	49.7
Cys.	+	+	+	+	+	+	+	+
Val.	26.0	29.4	27.8	41.1	20.5	24.4	21.0	33.8
Met.	+	+	+	+	+	+	+	+
I-leu.	17.3	20.4	19.2	27.3	14.3	16.0	14.1	22.6
Leu.	36.6	38.0	38.1	56.5	29.7	31.5	27.6	42.2
Tyr.	9.2	9.0	9.7	14.0	7.9	7.5	7.2	9.6
Phe.	17.2	17.2	17.6	27.8	13.4	14.8	12.4	20.1

*

The light treatment was 2 days of 16 hr light (1500 ft-c) and 8 hr darkness.

mutant pellets were equal to those of the normal at 15.5° in the dark suggests that a qualitative difference may have existed in the protein from the leaves of the two lines.

By examination of the results from the light-treated seedlings (tables 15 and 16) it may be seen that light had an even stronger differential effect on the two lines than did temperature. This is particularly noticeable in the amide contents. At 15.5° the mutant accumulated 1.6 times as much alcohol extractable asparagine, and 3.2 times as much glutamine. At 33.5° the differences were accentuated and these respective values become 6.2 and 4.5. Light was highly stimulatory to glutamine production in leaves of both barley lines, which is in agreement with previous findings (Kretovich, 1959). It was also effective in promoting total asparagine utilization at both 15.5 and 33.5° in the normal, but in the mutant at 15.5° the main effect appeared to be a conversion of alcohol non-extractable asparagine to the extractable form. In the dark the higher temperature promoted a very high accumulation of the non-extractable form in both lines; at the same time the extractable form decreased (table 15, asparagine values; table 16, aspartic acid values).

The total amino acid composition of the pellets from both experiments are given in the appendix, tables ii and iii, and a comparison of the composition of the leaf pellets from the second experiment is given in table iv. This latter table is calculated as per cent of leucine. Also included in table iv is a comparison of the ungerminated-seed protein of the two lines. Except for the somewhat higher lysine and arginine content of the mutant already

mentioned, little variation in amino acid composition existed between the mutant and normal leaf. Although all of the acids measured were present in higher quantities in the mutant seed than in the normal, it can be calculated that amide nitrogen (NH_4^+), aspartic acid and isoleucine showed the greatest percentage difference (μ moles acid in mutant/g seed $\times 100 \div \mu$ moles of same acid in normal/g seed). The higher protein content of mutant seeds indicated in the amino acid analyses was substantiated by micro Kjeldahl measurements of nitrogen (AOAC, 1950). The per cent nitrogen by weight was 2.89 in the mutant and 2.68 in the normal.

Table 17 is the result of the summation of the values obtained for each amino acid in all fractions, the amides being included in the appropriate acid values. These results, therefore, represent the total residues from the seedlings except for those which were in the peptide form and extracted with 95% ethanol (see appendix for example calculation). Spot-checks carried out by subjecting the alcohol extracts to hydrolysis with constant-boiling HCl indicated the presence of substantial amounts of peptides containing proline and glycine. The amount of these two acids in the peptide form was about equal to that in the free state. Peptides containing the other neutral and acidic amino acids were present in low amounts, usually in the range of 5 to 15% of the free acids.

In the first experiment only the pellets from the fourth, fifth, and ninth days were analysed, consequently data for the total residues was obtained only on these days. In general the changes in total residues were similar to those noted by Folkes and Yemm (1958),

Table 17

Experiments 1 and 2

Total* Amino Acid Residues in the Seedlings at Various Stages of Development. The 7-day-old Material was Grown at 24.0° in the Dark for 5 Days Prior to Transfer to the Conditions Noted

		(μ moles/g seed)							
					15.5° dark	33.5° dark	15.5° light**	33.5° light**	
Age (days)		0	4	5	7	7	7	7	9
Aspartic	N	65.2	90.8	136.9	182.9	229.0	162.2	115.1	288.7
	M	74.8	110.5	160.8	224.0	225.0	202.3	199.5	275.9
Threonine	N	45.4	40.2	39.0	50.9	49.4	48.8	53.5	45.3
	M	49.8	43.4	46.6	49.5	46.5	47.3	55.0	43.2
Serine	N	67.6	49.9	49.2	61.4	57.2	59.7	60.0	55.6
	M	70.0	56.0	55.8	62.3	55.1	61.8	61.8	54.7
Glutamic	N	289.5	169.3	125.7	120.7	95.5	116.2	108.5	75.2
	M	310.4	183.0	136.4	122.6	90.5	134.0	114.3	75.3
Proline	N	195.2	86.4	53.4	51.7	41.9	49.4	55.9	31.8
	M	210.2	89.9	62.4	45.8	37.8	44.3	47.1	32.8
Glycine	N	82.9	69.9	67.0	69.5	74.9	86.6	93.3	59.6
	M	88.4	74.2	81.2	78.3	72.1	77.4	82.0	59.3
Alanine	N	68.3	71.0	77.4	100.5	103.6	98.7	106.5	78.6
	M	76.0	78.0	87.6	99.7	98.9	93.1	102.3	75.3
Valine	N	64.8	57.7	54.4	68.5	67.1	62.4	64.9	56.3
	M	71.6	59.1	64.5	67.1	66.8	61.6	67.0	58.2
Isoleucine	N	44.2	38.8	34.0	43.9	43.0	42.0	42.7	32.0
	M	51.1	37.6	42.8	44.0	39.2	40.8	40.8	32.7
Leucine	N	96.3	76.8	62.9	79.7	69.0	75.2	84.3	49.2
	M	107.2	78.2	80.0	77.6	62.9	70.4	71.8	50.1
Tyrosine	N	25.9	22.5	19.0	19.7	19.3	19.8	21.7	16.1
	M	28.2	22.2	23.1	19.5	19.1	19.0	18.2	16.0
Phenylalanine	N	56.5	31.6	29.8	36.4	31.5	34.1	40.6	24.6
	M	59.2	38.9	37.9	33.7	30.0	29.9	32.9	24.8

* See appendix for example calculation.

** The light treatment was 16 hr light (1500 ft-c) and 8 hr darkness.

although definite comparisons cannot be made because of different experimental conditions. Greatest changes were in proline and glutamic acid, which declined rapidly, and in aspartic, which increased rapidly. All the amino acids measured except aspartic and alanine decreased when the seedlings were held for 9 days in the dark. Remarkable similarity existed in the quantity of total residues of each acid in the normal and mutant on the ninth day. It is apparent that light at 33.5° stimulated the production of most of the other amino acids at the expense of asparagine residues, especially in the normal barley line.

III. Ribonucleic Acid of Barley Leaves

The isolation of rRNA using the phenol extraction method yielded a product of high purity, although some variation occurred as noted in table 18. Contaminating substances, making up 35% of the extract in the 5-day-old normal samples, did not interfere with precise measurements of the nucleotides after chromatography. Typical absorption spectra of the nucleotides from the extracts are given in figure 3.

The nucleotide composition of the leaf rRNA of the two barley lines appears to differ very little (table 18). The major difference is in the quantity of Gp, resulting in a higher purine/pyrimidine ratio in the mutant at the 5-day-old stage. This difference is small, however, (<5%), and may be within the range of accuracy of the method used for measurement. The total amount of rRNA was higher in the normal at both ages tested. As will be mentioned later however, quantitative extraction of rRNA by this procedure is doubtful.

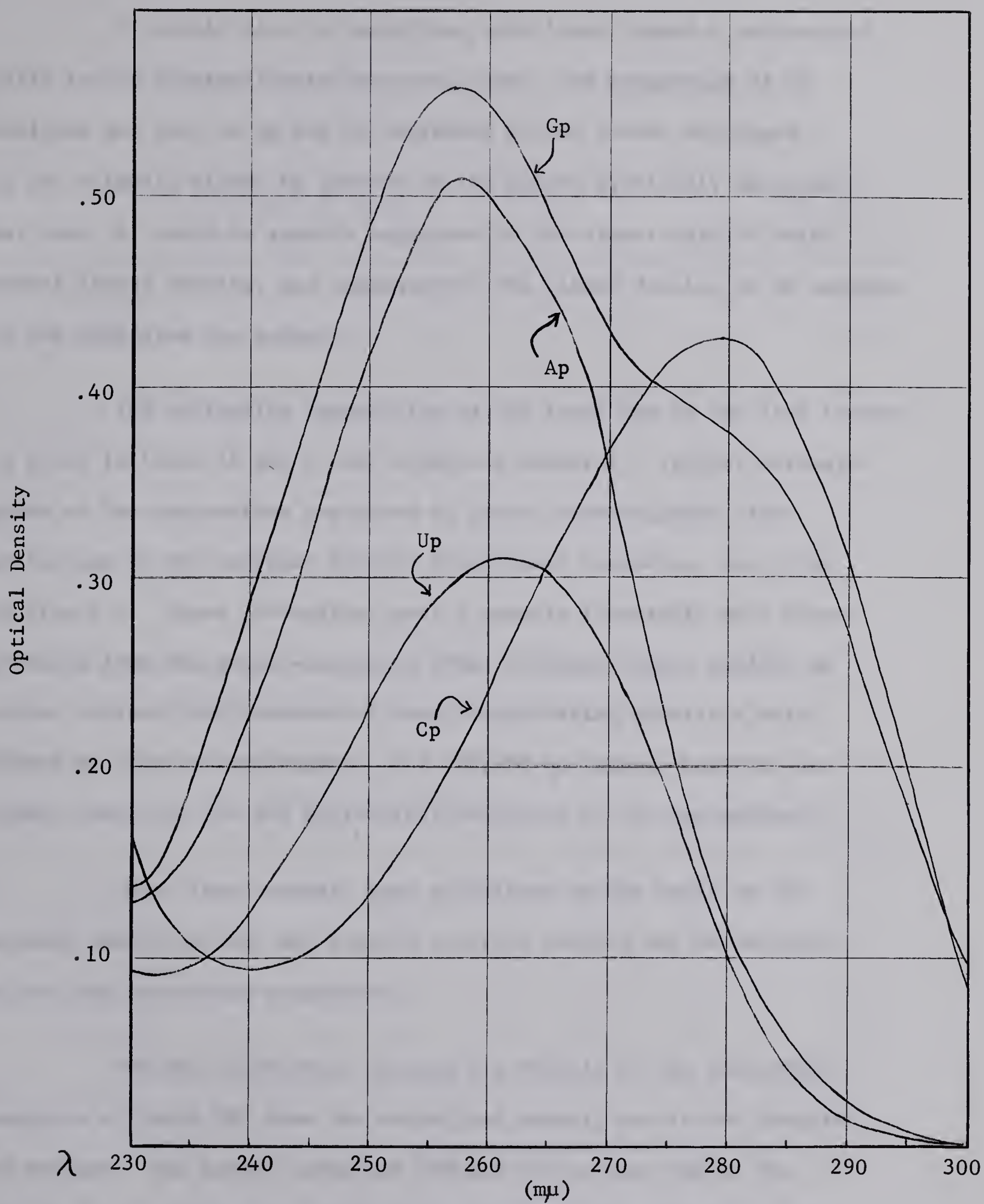


Fig. 3. Spectra of nucleotides isolated by paper chromatography of hydrolyzates of rRNA extracted by the phenol method.

It should also be noted that both lines showed a substantial shift in the 6-amino/6-keto ratio with age. The proportion of Gp declined and that of Ap and Cp increased as the leaves developed. If the slightly higher Gp content of the mutant previously mentioned was real, it could be readily explained by the slower rate at which mutant leaves develop, and consequently the slower decline in Gp content of the rRNA from the mutant.

The nucleotide composition of the total RNA in the leaf tissues is given in table 19 for 3 and 12-day-old material. Typical chromatograms of the nucleotides separated by paper chromatography after extraction by the modified Schmidt-Thannhouser procedure, are given in figure 4. These absorption spectra compare favourably with those produced from the phenol-extracted rRNA, although higher 230/260 μ ratios indicate the presence of some contaminating materials which absorb at shorter wavelengths. The 260/280 μ ratios, however, are almost identical for the nucleotides extracted by the two methods.

Thus since contents were calculated on the basis of the optical density at 260 μ equally accurate results may be obtained by the two extraction procedures.

The only difference between the results of the nucleotide analysis of total RNA from the mutant and normal, was in the quantity of extract. The mutant contained 79% and 73% as much RNA as the normal on the third and twelfth days, respectively. The base ratios purine/pyrimidine, and 6-amino/6-keto ratios were almost identical. These base ratios differ from those reported by Zscheile and Murray

Table 18

rRNA Content of Mutant and Normal Barley Leaf Tissue
(Phenol Extraction Method)

		moles/100 moles				Purity of powder	pu/py	6-amino /6-keto	mg nucleo- tides /g dry leaf
		Ap	Gp	Cp	Up				
Normal	5-day-old	25.5	31.5	22.5	20.5	65%	1.13	.92	8.69
	12-day-old	27.4	27.3	25.7	19.5	91%	1.21	1.13	6.11
Mutant	5-day-old	25.2	33.1	22.3	19.3	91%	1.40	.91	7.42
	12-day-old	26.8	28.2	25.7	19.3	91%	1.22	1.11	5.55

Table 19

Total RNA Content of Mutant and Normal Barley Leaf Tissue
(Modified Schmidt-Thannhouser Extraction)

		moles/100 moles				pu/py	6-amino /6-keto	mg nucleo- tides/g dry leaf
		Ap	Gp	Cp	Up			
Normal	3-day-old	24.5	35.2	21.3	19.0	1.48	85	23.36
	12-day-old	24.7	33.8	22.0	19.5	1.41	88	14.44
Mutant	3-day-old	24.2	35.0	21.7	19.1	1.45	85	18.56
	12-day-old	24.2	34.2	22.7	18.9	1.40	88	10.56

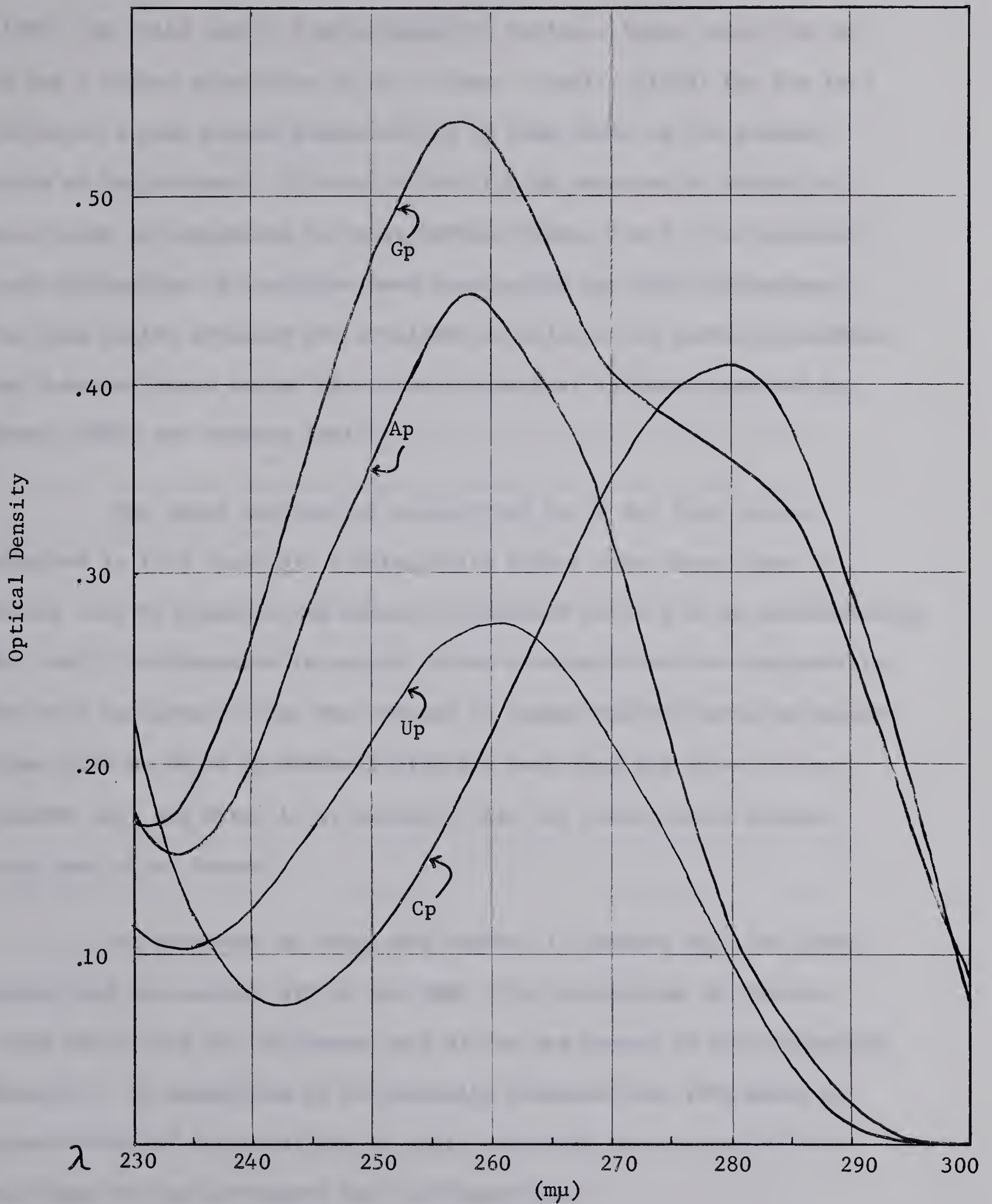


Fig. 4. Spectra of nucleotides isolated by paper chromatography of hydrolyzates of leaf tissue prepared by the modified Schmidt-Thannhouser method.

(1963) for Atlas barley leaves mainly in having a lower proportion of Up and a higher proportion of Ap. Thomas' results (1956) for rye leaf indicated a much higher proportion of Up than found in the present study of barley leaf. In view of the high Up reported by Thomas in wheat germ in comparison to other workers (Lane, 1964) it is possible that differences in technique were responsible for this discrepancy. The base ratios obtained for total RNA as well as the purine/pyrimidine and 6-amino/6-keto ratios were almost identical to those reported by Reddi (1959) for tobacco leaf.

The total quantity of nucleotides per g dry leaf tissue reported in this study are substantially higher than those found in barley leaf by Zscheile and Murray (calculated to be 8.23 mg nucleotides/g dry leaf). Differences in age of leaves used may have been responsible for this difference since the content in normal barley leaves decreased from 23.36 to 14.44 mg nucleotides/g dry leaf from the third to the twelfth day, and since it is probable that the above-quoted authors used even older leaves.

On the basis of total RNA content it appears that the phenol method did not extract all of the rRNA. The percentage of rRNA to total RNA was 42 for the normal and 53 for the mutant in the 12-day-old material. In comparison it is generally reported that rRNA makes up about 80-85% of the total RNA in cells, although no estimate of this was found in the literature for leaf material.

CONCLUSIONS

It is generally recognized that high light intensity destroys chlorophyll in plants. If the expression of the mutant gene (yv_2) was due to the production of a more light-sensitive form of chlorophyll holochrome in the mutant than normal, it would be expected that high light intensity would have a greater deleterious effect on the mutant than on the normal, regardless of temperature. Since the mutant was less sensitive to light intensity than the normal at 29.0 (fig 2), it appears that the mutant has not resulted in such a change in the chlorophyll holochrome.

The Q_{10} values for chlorophyll accumulation in the mutant at low temperatures indicated that a highly temperature-sensitive reaction was involved in the mutation. According to Klein (1960) grana formation has a large temperature coefficient.

Since the effect of light of different wavelengths must be considered as a difference in the qualitative utilization of light by the mutant and normal, this effect may be interpreted as being the result of a change in the physical structure of the absorbing unit — the grana. Furthermore, the presence of the same pigments in the mutant as in the normal (Maclachlan and Zalik, 1963) suggests that the change in the grana structure was due to an alteration in a moiety other than the pigments themselves. The fact that all pigments measured were affected in the same way by the chemicals which showed a differential action on the normal and mutant substantiates the above suggestion.

The mutation seriously affected the metabolism of amino compounds when the plants were grown in the light, and to a lesser extent when grown in the dark. The greatest effect was on the utilization of amide, especially asparagine, for the synthesis of other nitrogen containing compounds. The 20% reduction of amino acids in the leaf pellets of dark-grown mutant seedlings compared to those of the normal (table 16) indicated that the mutant effect was operative in the dark, even though under these environmental conditions the free amino acid levels did not differ substantially in the two lines. That the mutant effect acted in darkness was also indicated in previous results on protochlorophyll formation (Maclachlan and Zalik, 1963). Whether the fault in leaf protein synthesis was a general phenomenon or whether it involved a certain type of protein was not definitely determined. However, since cytoplasmic protein has a higher lysine content than chloroplastic protein (Yemm and Folkes, 1953), and since lysine content of mutant leaves was nearly always higher than the other acids in relation to the content of normal leaves, this suggests that the mutation may have affected only chloroplastic protein. It is possible, therefore, that the mutation has resulted in the same disruption of protein synthesis as iron deficiency (Perur et al., 1961). Furthermore since the mutant seeds contained more protein than did the normal, synthesis of this fraction of protein was not hindered by the mutation, again indicating that specific protein synthesis rather than general protein synthesis was disrupted in the mutant.

The amount of rRNA relative to the total RNA extracted from the two barley lines suggests two alternatives: (1) That rRNA of the

mutant leaves was more easily extracted by the phenol procedure or (2) A considerably smaller fraction of the total RNA is made up of sRNA and mRNA in the mutant than in the normal. These alternatives are based on the following reasoning. If the content of rRNA is taken as 80% of the total RNA, then in 12 day old normal leaf tissue there should have been 11.55 mg/g of rRNA. The extracted quantity of rRNA in this tissue was 6.11 mg/g or 53%. By the same calculation the mutant should have contained 8.45 mg/g tissue, and that which was extracted (5.55 mg/g) represented 66% of the total rRNA, or a considerably greater proportion than in the normal extract. These figures suggest a difference in the state of rRNA in the two barley lines.

The second alternative is based on the assumption that the phenol extraction method extracted the same proportion of the total rRNA from the two barley lines. Again assuming that 80% of the total RNA is made up of rRNA in the normal, 53% of the rRNA was extracted. If this same proportion of rRNA in the mutant was extracted, it can be calculated that 10.47 mg/g leaf of rRNA was present in the mutant. Since the total extracted was only 10.56 mg/g very small amounts of sRNA and mRNA could be present.

Further experiments are necessary, including the measurement of sRNA and mRNA, in order that a choice of the alternatives may be made.

Current theories concerning the action of kinins on plants seem to be inadequate to explain all of their observed effects. The suggestion that maintenance of chlorophyll content through the inhibition

of RNAase and the consequent enhancement of protein levels in treated tissues (Richmond and Lang, 1957) does not appear to explain the specific toxic effects obtained in other cases, in spite of the concurrent changes in RNA and protein in treated plants (Tsujita, 1964). For instance, such a theory would not appear to explain why N⁶-benzyladenine caused a significant reduction in chlorophyll in the normal barley line but not in the mutant.

In view of the varying results it may be suggested that at low concentrations kinin enters more or less specifically into the mRNA responsible for RNAase synthesis, but at higher concentrations incorporation into other mRNA's is increased. Thus at low concentrations of kinin there may be a beneficial effect on general RNA accumulation, and incidentally on chlorophyll holochrome, by combining irreversibly with RNAase, but at higher concentrations there is an inhibition of synthesis of active mRNA responsible for the production of essential proteins. The reduction of chlorophyll in normal barley treated with N⁶-benzyladenine could therefore have been due to an inhibition of synthesis of chlorophyll holochrome protein through suppression of the formation of the corresponding active mRNA. The complete lack of any effect on the mutant by either kinetin or N⁶-benzyladenine would seem to indicate that the mutation has resulted in an alteration in the process of kinin incorporation into mRNA responsible for the synthesis of chlorophyll holochrome protein. This alteration itself could have resulted in a protein whose rate of production, or whose rate of incorporation into the holochrome was reduced.

Although there are still areas of metabolism which have not been investigated, such as lipid, sugars and other carbohydrates, it is suggested that further studies concerning the effects of kinins on the metabolism of RNA, protein and chlorophyll, would be most fruitful in obtaining information on the action of the mutant gene.

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Plant Species	Sample	Adenine	Guanine	Cytosine	Uracil
Spinach	1	1.25	1.10	1.15	1.10
Spinach	2	1.20	1.05	1.10	1.05
Spinach	3	1.15	1.00	1.05	1.00
Spinach	4	1.10	0.95	1.00	0.95
Spinach	5	1.05	0.90	0.95	0.90
Spinach	6	1.00	0.85	0.90	0.85
Spinach	7	0.95	0.80	0.85	0.80
Spinach	8	0.90	0.75	0.80	0.75
Spinach	9	0.85	0.70	0.75	0.70
Spinach	10	0.80	0.65	0.70	0.65
Spinach	11	0.75	0.60	0.65	0.60
Spinach	12	0.70	0.55	0.60	0.55
Spinach	13	0.65	0.50	0.55	0.50
Spinach	14	0.60	0.45	0.50	0.45
Spinach	15	0.55	0.40	0.45	0.40
Spinach	16	0.50	0.35	0.40	0.35
Spinach	17	0.45	0.30	0.35	0.30
Spinach	18	0.40	0.25	0.30	0.25
Spinach	19	0.35	0.20	0.25	0.20
Spinach	20	0.30	0.15	0.20	0.15
Spinach	21	0.25	0.10	0.15	0.10
Spinach	22	0.20	0.05	0.10	0.05
Spinach	23	0.15	0.00	0.05	0.00
Spinach	24	0.10	0.00	0.00	0.00
Spinach	25	0.05	0.00	0.00	0.00
Spinach	26	0.00	0.00	0.00	0.00
Spinach	27	0.00	0.00	0.00	0.00
Spinach	28	0.00	0.00	0.00	0.00
Spinach	29	0.00	0.00	0.00	0.00
Spinach	30	0.00	0.00	0.00	0.00

Table 1. Nucleotide composition of RNA in spinach leaves. Values are expressed as percentages of total nucleotides.

APPENDIX

Table i

Free Amino Acids of Entire Seedlings Germinated 5 Days in Darkness
at 24.0°, Followed by 2 Days Under Conditions Noted
at the Top of Each Column

		(μ moles/g seed)				
		(Exp. 1)	15.5° dark	33.5° dark	15.5° light*	33.5° light*
Age (days)		5	7	7	7	7
Aspartic	N	2.65	5.87	6.33	5.99	5.38
	M	2.08	7.99	6.57	10.24	9.98
Threonine	N	5.33	6.36	10.09	4.79	4.99
	M	6.13	9.83	10.57	8.29	10.90
Serine	N	5.84	8.21	9.25	7.96	5.20
	M	4.61	12.48	10.28	13.00	9.63
Glutamic	N	6.34	12.79	14.25	15.50	12.34
	M	4.66	17.51	13.35	13.12	12.83
Glycine	N	2.59	2.59	3.35	2.46	1.39
	M	2.18	3.82	4.15	3.26	2.74
Alanine	N	11.10	18.01	29.02	13.48	11.88
	M	9.36	23.47	29.63	17.78	18.80
Valine	N	16.96	15.85	20.78	8.52	5.34
	M	16.96	20.57	23.82	14.66	11.89
Isoleucine	N	10.77	8.38	10.41	5.26	2.57
	M	11.61	12.47	11.12	9.48	5.03
Leucine	N	12.61	8.76	7.89	4.52	2.34
	M	13.31	13.06	8.19	9.22	4.48
Tyrosine	N	5.64	2.75	5.46	1.54	1.79
	M	4.98	3.93	5.97	2.36	2.83
Phenylalanine	N	7.06	3.18	3.70	1.46	1.33
	M	6.43	4.49	4.29	1.99	1.39
Asparagine	N	54.76	58.86	49.88	63.65	23.98
	M	41.24	101.13	53.77	98.14	95.87
Glutamine	N	32.04	9.49	4.69	16.37	8.10
	M	33.16	26.05	7.50	40.88	20.84

* The light treatment was 16 hr light (1500 ft-c) and 8 hr darkness.

Table ii

Experiments 1 and 2

Amino Acid Composition of Total Protein from Normal Seedlings at Various Stages of Development in the Dark at 24.0^o, or from the Fifth Day Onward as Indicated at the Top of Each Column

Age (days)	(μ moles/g seed)							
			15.5 ^o		33.5 ^o		24.0 ^o	
			dark		dark		light*	
	0	4	5	7	7	7	7	9
Lysine	34.0			47.2	47.9	48.8	56.5	34.4
Histidine	22.2			16.3	14.2	15.4	16.8	11.4
Ammonia	269.2			144.3	172.5	707.0	98.1	90.6
Arginine	40.6			33.2	31.2	34.9	37.1	21.8
Aspartic	65.2	61.2	79.5	118.2	172.8	92.6	85.7	82.8
Threonine	45.4	34.8	33.5	44.5	39.3	44.0	48.5	28.7
Serine	67.6	44.5	43.4	53.2	48.0	51.7	54.8	35.1
Glutamic	289.5	126.8	74.4	98.4	76.6	84.4	88.0	45.8
Proline	195.2	72.1	44.4	51.7	41.9	48.8	55.3	30.3
Glycine	82.9	67.8	64.4	80.9	71.6	84.1	91.9	54.4
Alanine	68.3	64.1	66.3	82.5	74.6	106.4	94.6	56.1
Valine	64.8	43.5	37.5	52.6	48.0	53.9	59.5	30.6
Isoleucine	44.2	29.1	23.2	35.5	32.6	36.7	40.1	79.1
Leucine	96.2	62.3	50.3	71.5	61.2	70.7	82.0	41.1
Tyrosine	25.9	16.6	13.4	17.0	13.9	18.3	19.9	9.6
Phenylalanine	56.5	28.5	22.7	33.2	27.8	32.6	39.3	18.4

* The light treatment was 2 days of 16 hr light (1500 ft-c) and 8 hours darkness.

Table iii

Experiments 1 and 2

Amino Acid Composition of Total Protein from Mutant Seedlings at Various Stages of Development in the Dark at 24.0°, or from the Fifth Day Onward as Indicated at the Top of Each Column.

Age (days)	(μ moles/g seed)							
	15.5° dark				33.5° dark			
	15.5° light				15.5° light			
	0	4	5	7	7	7	7	9
Lysine	37.1			49.5	45.5	46.8	50.9	34.9
Histidine	23.6			15.4	14.1	14.6	15.3	11.5
Ammonia	317.6			125.4	187.8	101.9	115.9	117.0
Argenine	42.9			33.4	30.8	32.6	35.2	21.2
Aspartic	74.8	75.5	117.5	115.0	164.7	93.9	93.6	96.6
Threonine	49.8	36.5	40.7	39.7	35.9	39.0	44.1	27.8
Serine	70.0	49.2	51.1	49.8	44.9	48.8	52.2	36.6
Glutamic	310.4	125.0	98.6	79.0	69.8	80.0	80.7	42.8
Proline	210.2	74.4	54.6	43.5	37.8	42.8	46.2	31.0
Glycine	88.4	71.6	79.0	74.5	68.0	74.1	79.2	53.9
Alanine	76.0	69.9	78.3	76.2	69.5	75.3	83.5	56.2
Valine	71.6	43.3	47.6	46.5	43.1	46.9	55.1	30.5
Isoleucine	51.1	27.5	31.2	31.5	28.2	31.4	35.8	19.4
Leucine	107.2	62.6	66.7	64.6	54.7	61.2	67.3	41.8
Tyrosine	28.2	15.7	18.2	15.6	13.1	16.7	15.3	9.7
Phenylalanine	59.2	29.9	31.5	29.2	25.6	28.0	31.5	19.0

* The light treatment was 2 days of 16 hr light (1500 ft-c) and 8 hrs darkness.

Table iv

Experiment 2

Amino Acid Composition of Ungerminated Seed and of Leaf Pellets
from 7-day-old Barley Seedlings Germinated 5 Days in the Dark at 24°,
then 2 Days at the Conditions Indicated at the Top of each Column.
Expressed as % of Leucine.

	Normal Leaf				Mutant Leaf				Seed	
	15.5° dark	33.5° dark	15.5° light*	33.5° light*	15.5° dark	33.5° dark	15.5° light*	33.5° light*	N	M
Lys.	63	84	72	65	83	90	84	72	35	35
His.	21	23	20	20	23	25	22	22	23	22
NH ₄ ⁺									280	296
Arg.	43	52	48	42	53	55	53	50	42	40
Asp.									68	70
Thr.	63	67	61	60	61	66	64	63	47	46
Ser.	73	81	68	64	76	81	79	72	70	65
Glu.	129	131	114	110	118	126	127	115	301	290
Pro.	76	65	62	65	67	67	63	65	203	196
Gly.	111	118	112	112	110	118	118	111	86	82
Ala.	116	126	115	115	117	123	121	118	71	71
Val.	71	77	73	73	69	77	76	80	67	67
I-leu.	47	54	51	48	48	51	51	54	48	48
Leu.	100	100	100	100	100	100	100	100	100	100
Tyr.	25	24	26	25	26	24	26	23	27	26
Phe.	47	45	46	49	45	47	45	48	59	55

* The light treatment was 2 days of 16 hr light (1500 ft-c) and 8 hr darkness.

Table v

Experiment 2

Amino Acid Composition of Roots plus Endosperm of 7-day-old Barley
Seedlings Germinated 5 Days in Darkness at 24.0^o, then 2 Days
at the Conditions Indicated at the Top of Each Column

	(μ moles/g seed)							
	Normal				Mutant			
	15.5 ^o dark	33.5 ^o dark	15.5 ^o light*	33.5 ^o light*	15.5 ^o dark	33.5 ^o dark	15.5 ^o light*	33.5 ^o light*
Lys.	24.0	15.9	21.4	19.9	25.0	17.2	23.6	20.5
His.	8.6	5.3	7.8	5.6	8.5	6.2	8.6	6.2
NH ₄ ⁺	72.4	44.0	49.9	32.6	56.9	69.4	47.0	46.9
Arg.	17.6	11.3	16.5	13.4	17.6	13.3	18.1	14.2
Asp.	52.4	31.1	44.2	24.8	47.1	41.4	50.4	34.5
Thr.	21.3	13.7	20.9	14.6	21.5	15.2	21.5	17.6
Ser.	26.4	17.2	26.0	18.4	27.4	19.2	27.2	21.8
Glu.	51.1	26.9	40.9	25.8	43.8	30.1	45.0	32.2
Pro.	25.5	17.3	25.3	18.4	23.7	16.8	25.4	18.9
Gly.	40.4	26.9	41.4	28.9	41.8	30.7	41.6	32.2
Ala.	39.9	26.9	41.6	29.8	41.4	30.7	41.8	33.8
Val.	26.6	17.0	26.1	18.4	26.0	18.7	25.9	21.3
I-Leu.	18.2	12.3	17.5	12.9	17.2	12.2	17.3	13.2
Leu.	34.3	23.1	32.6	25.5	34.9	23.2	33.6	25.2
Tyr.	7.8	4.9	8.6	5.8	7.7	5.6	9.4	5.7
Phe.	16.0	10.6	15.0	11.5	15.7	10.8	15.6	11.4

* The light treatment was 2 days of 16 hr light (1500 ft-c) and 8 hr darkness.

Sample Calculation of Values in Table 17

Total aspartic residues in the normal line on seventh day in dark
at 15.5° = 182.9.

Free aspartic in leaf = 3.32

Free aspartic in root + endosperm = 2.55

Free asparagine in leaf = 42.40

Free asparagine in root + endosperm = 16.46

Aspartic in hydrolyzed leaf pellet = 65.80

Aspartic in hydrolyzed root + endosperm = 52.40

Total = 182.93

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